MicroRNA 223-dependent expression of LMO2 regulates normal erythropoiesis

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ABSTRACT

Background

MicroRNAs are small non-coding RNAs that regulate gene expression through mRNA degradation or translational inhibition. MicroRNAs are emerging as key regulators of normal hematopoiesis and hematologic malignancies. Several miRNAs are differentially expressed during hematopoiesis and their specific expression regulates key functional proteins involved in hematopoietic lineage differentiation. This study focused on the functional role of microRNA-223 (miR-223) on erythroid differentiation.

Design and Methods

Purified cord blood CD34$^+$ hematopoietic progenitor cells were grown in strictly controlled conditions in the presence of saturating dosage of erythropoietin to selectively induce erythroid differentiation. The effects of enforced expression of miR-223 in unilineage erythroid cultures were evaluated in liquid phase culture experiments and clonogenic studies.

Results

In unilineage erythroid culture of cord blood CD34$^+$ hematopoietic progenitor cells miR-223 is down-regulated, whereas LMO2, an essential protein for erythroid differentiation, is up-regulated. Functional studies showed that enforced expression of miR-223 reduces the mRNA and protein levels of LMO2, by binding to LMO2 3' UTR, and impairs differentiation of erythroid cells. Accordingly, knockdown of LMO2 by short interfering RNA mimics the action of miR-223. Furthermore, hematopoietic progenitor cells transduced with miR-223 showed a significant reduction of their erythroid clonogenic capacity, suggesting that downmodulation of this miRNA is required for erythroid progenitor recruitment and commitment.

Conclusions

These results show that the decline of miR-223 is an important event for erythroid differentiation that leads to the expansion of erythroblast cells at least partially mediated by unblocking LMO2 protein expression.

Key words: hematopoietic progenitors cells, erythroid differentiation, microRNA, transcription factors.

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Introduction

Erythropoiesis, the differentiation of hematopoietic stem cells (HSCs) into erythroid lineage, involves a series of intermediate differentiation stages including the burst forming unit-erythroid (BFU-E), colony forming unit-erythroid (CFU-E), and erythroid precursor cells. This process involves complex interactions of transcription factors that modulate the expression of downstream genes and mediate proliferation and differentiation signals.[1,2]

LMO2 (LIM-only protein 2, RBNT2), is a member of the LIM-only class of transcriptional co-regulators that consist of little more than two closely spaced cysteine-rich LIM domains.[3] LMO2 is an important regulator of hematopoietic stem cell development and erythropoiesis, as mice lacking this gene show defects in blood formation as well as in the formation of fetal erythrocytes.[4] LMO2 has been demonstrated to be part of a multimeric transcriptional complex, which includes the basic helix-loop-helix proteins SCL and E2A, the LIM-domain binding protein 1 (Ldb-1) and GATA1.[5] Together these proteins form a DNA binding complex regulating the expression of genes involved in erythroid differentiation.[6-9] Furthermore, it has been shown that enforced expression of LMO2 in multipotent progenitor cells increased erythroid differentiation enhancing transcription of erythroid genes.[10]

Beside transcription factors, hematopoietic differentiation is determined by post-transcriptional regulators including microRNA (miRNAs).

MiRNAs are a class of small non-coding RNAs[11] that regulate gene expression through degradation or translational repression of target mRNAs.[12]

Previous findings showed that several miRNAs are differentially expressed during hematopoiesis and their specific expression may modulate hematopoietic lineage differentiation, indicating that miRNAs have a functional role in normal and malignant hematopoiesis.[13-15]

The commitment of pluripotent precursors to the erythroid lineage and the differentiation of committed erythroid progenitors into erythrocytes are associated with the induction or repression of several miRNAs.[16-20] However, the majority of their target genes are predicted computationally and only a few targets have been validated so far, preventing delineation of miRNA-based control circuitries.

The role of microRNA-223 (miR-223) in hematopoiesis has been extensively analyzed. Particularly, several studies focused the attention on its function during granulopoiesis where it acts as a fine-tuner of granulocytic differentiation, maturation and function.[21-23] Expression profiling analysis indicates that miR-223 expression decreases as cells mature during monocytic,[23] erythroid[17] and mast-cell differentiation.[24] However, no functional studies have been performed to evaluate the role of miR-223 during monocytic, megakaryocytic and erythroid differentiation.

This study focuses on the functional role of miR-223 in erythroid differentiation/maturation, as evaluated in unilineage erythroid culture of cord blood (CB) CD34$^+$ hematopoietic progenitor cells (HPCs). Our results show that miR-223 is markedly down-regulated during erythropoiesis and this decline is required for erythrocyte proliferation and differentiation at progenitor and precursor level. Accordingly, the downmodulation of miR-223 appears to be a relevant step to promote erythropoiesis favoring translation of key functional target protein(s). Consistent with this hypothesis we identified LMO2 protein as a critical target of miR-223.

Design and Methods

Cord blood human progenitor cells purification and cultures

Collection of cord blood (CB), isolation of CD34$^+$ cells, unilineage culture and morphology analysis were performed as previously described.[25] Briefly, CB was obtained after informed consent from healthy full-term placentas according to institutional guidelines. Human CD34$^+$ cells were purified from CB by positive selection using the midi-MACS immunomagnetic separation system according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladabach, Germany). CD34$^+$ progenitors were cultured in serum-free medium in the presence of various recombinant human cytokine combinations.

For erythroid unilineage culture, medium was supplemented with 0.01U/mL IL-3, 0.001 ng/mL GM-CSF (PeproTech Inc. Rocky Hill, NJ, USA) and 3 U/mL erythropoietin (Amgen Thousand Oaks, CA, USA). For megakaryocytic unilineage culture, medium was supplemented with 100 ng/mL thrombopoietin (PeproTech). For granulocytic unilineage culture, medium was supplemented with 1 U/mL IL-3, 0.1 ng/mL GM-CSF combined with plateau level of G-CSF (500 U/mL) (PeproTech). For monocytic unilineage culture, medium was supplemented with 1 ng/mL IL-6, 100 ng/mL Flt3-ligand, combined with plateau level M-CSF (50 ng/mL) (PeproTech).

For morphological analysis, cells were smeared on glass slides by cytospin centrifugation, stained with May-Grünwald-Giemsa and analyzed at 400x or 600x magnification under a microscope (Eclipse 1000, Nikon, Tokyo, Japan) equipped with a digital camera.

Clonogenetic assay

For clonogenesis, 3×10^2 cells were plated in duplicate in 0.9% methylcellulose containing serum free medium, saturating concentrations of erythropoietin (3 U/mL), 0.01 U/mL IL-3 and 0.001 ng/mL GM-CSF.

Plasmids

psiCHECKTM-3'UTR plasmid: to determine the binding of miR-223 to LMO2 its 3' UTR (NM_005574) was amplified by PCR using the primers 5'-GATGATATAGGCCCGAGTCC-3', 5'-TCTACACACGACAAATACTT-3' and cloned into XhoI/NotI sites of the psiCHECKTM-2 vector (Promega, Madison, WI, USA) to perform luciferase assay.[26] This vector allows simultaneous expression of renilla and firefly luciferase.[27] The LMO2 3'UTR was cloned downstream the renilla

luciferase allowing the expression of a renilla transcript with the 3'UTR. Renilla luciferase activity is then used to assess the effect of the 3'UTR on transcript stability and translation efficiency. The second reporter, firefly luciferase serves as control.

The mutations of the site of perfect complementarity were introduced by PCR mutagenesis using the QuickChange Site-Directed Mutagenesis kit protocol (Stratagene, La Jolla, CA, USA) and were confirmed by DNA sequencing.

The oligonucleotides used to introduce mutations were: 5'-CAGCCCATCCATAGTAggccgaACCGAA TGATTAGCAGAAG-3', 5'-CTTCTGCTAATCATT CGGTtcggccGGCCTACTATGGATGGGCTG-3'. Lentiviral vector: the lentiviral vector encoding miR-223 was generated as previously described.[21]

Oligonucleotides and transfection experiments

HeLa cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotics. Cells were co-transfected with 100 ng of psiCHECK™-3'-UTR plasmid, 10 pmol of either a stability-enhanced non-targeting dsRNA control oligonucleotide or a stability-enhanced miR-223 (Dharmacon, Lafayette, CO, USA), all combined with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The activities of firefly and renilla luciferases in the cellular extracts were measured using the dual-luciferase reporter assay system (Promega). Briefly, after 48 h cells were washed, lysed and luciferin reagent were added according to the manufacturer's protocol (Promega). Luciferase protein catalyzes a light-emitting reaction when incubated with ATP and luciferin. Light emission was determined using a luminometer (Dynatech Laboratories, Chantilly, VA, USA). Relative luciferase activity was obtained by normalizing the activity of the renilla luciferase against the internal control of firefly luciferase.

CB $CD34^+$ progenitors cultured in erythroid medium were transfected on day 3. Cells were seeded (1.25×10^5 cells/mL) in antibiotic-free media and transfected with 160 nM of either a non-targeting control miRNA or miR-223 (Ambion, Austin, TX, USA) plus a FITC-conjugated double strand RNA (in a 10 to 1 proportion), all combined with Lipofectamine 2000 (Invitrogen). Human small interfering RNA against LMO2 were purchased by Ambion (ID: s8230 and s8232). Twenty-four hours after transfection, FITC positive cells were sorted by a FACSAria instrument (Becton Dickinson, Franklin Lakes, NJ, USA).

RNA extraction, Northern blot and real-time RT-PCR

Total RNA was obtained using Trizol reagent. For Northern blot RNA samples (25 mg each) were run on 15% acrylamide denaturing Criterion pre-cast gels (Bio-Rad, Hercules, Ca, USA) and then transferred onto Hybond-N+ membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). The hybridization was performed with specific probe, previously labeled with [γ]-^{32}PATP, at 37°C in 0.1% SDS/6x SSC overnight. Membranes were washed at room temperature twice with 0.1% SDS/2 x SSC. Human tRNA for initiator methionine (Met-tRNA) was used as loading control.

The probes used were:
miR 223 – 5'-GGGGTATTTGACAAACTGACA – 3'
Met-tRNA – 5'TGGTAGCAGAGGATGGTTTCGATC-CATCGACCTCTG-3'.

Blots were stripped at 65°C in 0.1% SDS/0.1 x SSC for 15 min and reprobed.

Real-time PCR for miR-223 was performed using TaqMan® MiRNA Assays protocol (Applied Biosystems, Foster City, CA, USA). Briefly, reverse transcriptase reaction was performed using 10 ng of DNAse treated total RNA and 50 nM miRNA specific stem-loop RT primers (P/N 4373075). Real-time PCR was performed using standard protocol. All reactions were run in duplicate. Normalization was performed by using RNU6B primer kit (P/N 4373381, Applied Biosystems). Relative expression was calculated with relative standard curves for both the miRNA of interest and the endogenous control.

Western blotting

Whole cell extracts were prepared as previously described.[28] Whole cell extracts were loaded onto a 12% SDS-PAGE, transferred onto Hybond-C paper (Amersham Biosciences), incubated with anti-LMO2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-actin (Oncogene Research, San Diego, CA, USA), anti-BCL2 (Santa Cruz Biotechnology) and detected using ECL detection kit (Pierce, Rockford, IL, USA). The expression levels were analyzed by the Scion Image Software (Scion, Frederich, MD, USA).

Infection

Infectious particles were produced as previously described.[21] Cells were infected by the spin inoculation method.[29] Concisely, cells were plated at 1×10^5/mL in lentiviral supernatant (>10^6 pfu/mL) in the presence of 4 μg/mL polybrene, and centrifuged at 32°C for 45 min. After spin inoculation the cells were incubated at 37°C 5% CO_2 for 1 h 15 min followed by plating in fresh media. GFP^+ cells were sorted 48h post-infection using FACSAria (Becton Dickinson).

Flow cytometry analysis

Cells were washed and incubated 30 min on ice in the dark with 3-5 mg/mL PE conjugated monoclonal antibody to Glycophorin A (GPA), CD34, CD41, CD15 and CD14 or isotype control (Pharmingen, San Diego, CA, USA). Cells were then washed and analyzed on a FACSCanto (Becton Dickinson). Non-viable cells were excluded by 30 mg/mL 7-amino-actinomycin D (7-AAD) (Sigma Aldrich, St. Louis, MO, USA). Cells with intact membranes prevent entry of the dye into the cells, subsequent binding to DNA and fluorescence emission. Acquisition and analysis were performed using FACS Diva software (Becton Dickinson).

Statistical analysis

Data are presented as mean values and error bars indicate SD. The groups were compared by two-way analysis of variance (ANOVA) using Bonferroni's test. $p<0.05$ was considered statistically significant.

Results

miR-223 expression in CB CD34⁺ HPCs unilineage differentiation

Unilineage cultures of cord blood CD34⁺ HPCs have been developed through erythroid (E),[28] megakaryocytic (MK),[30] granulocytic (G) and monocytic (Mo)[31] pathways. The CB CD34⁺ HPCs unilineage cultures recapitulate physiological hematopoiesis, allowing the analysis of 85-95% pure unilineage precursors starting from initial differentiation of HPCs until terminal maturation. In these serum-free liquid suspension culture, purified HPCs are induced to: (a) selective E growth by very low dosages of IL-3, GM-CSF, and a saturating erythropoietin (Epo) level, (b) unilineage MK growth by saturating dosage of thrombopoietin (Tpo), (c) unilineage G growth by low dose of IL-3 and GM-CSF combined with plateau level of G-CSF, and (d) unilineage Mo by low dose of IL-6 and saturating dosage of Flt-3 and M-CSF. In each type of culture cell growth is associated with a decrease in CD34⁺ positive cells and a parallel progressive increase in lineage-specific surface antigens (Figure 1).

These culture systems were used to analyze miR-223 expression in unilineage CB G, Mo, MK, and E cultures at sequential stages of differentiation and maturation.

The expression analysis of miR-223 was performed by Northern blot (Figure 2A, top) and real-time RT-PCR (qRT-PCR) (Figure 2A, bottom). As shown in Figure 2A, miR-223 is expressed in HPCs and its level strongly and progressively increased during G[23] and MK differentiation. In the Mo lineage the expression of miR-223 slightly decreases as cells differentiate, but it remains constantly expressed through monocytic terminal maturation. Conversely, in erythroid cultures miR-223 is strongly down-modulated and is essentially undetectable during all stages of E differentiation/maturation (Figure 2A). These results indicate that miR-223 markedly declines only during erythroid differentiation and maturation,

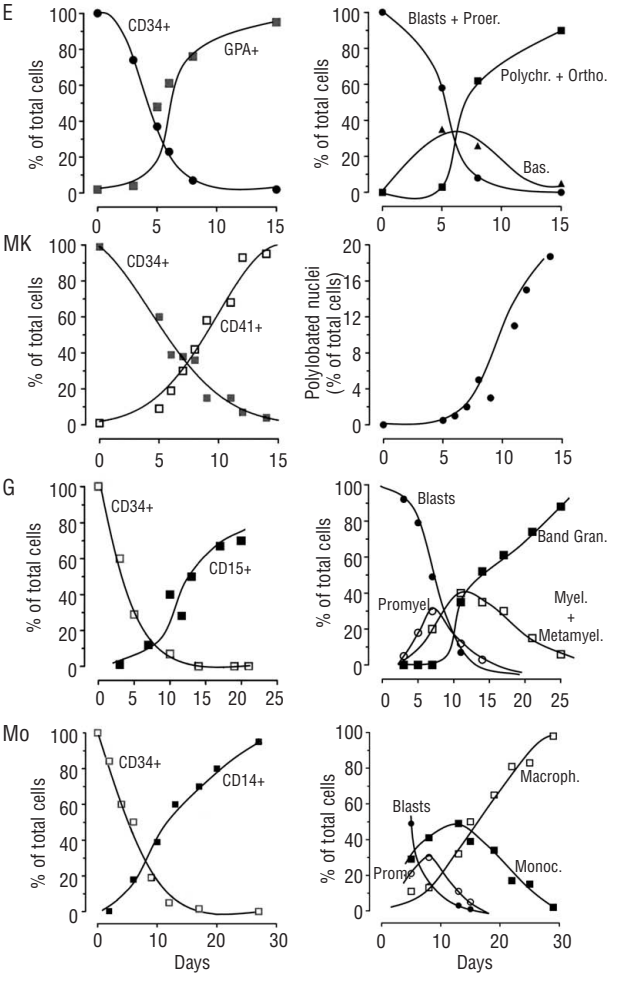

Figure 1. Growth and differentiation of cord blood (CB) CD34⁺ hematopoietic progenitor cells (HPCs) in unilineage cultures. (*left*) Percentage of GPA, CD41, CD15 and CD14 positive cells in unilineage erythroid, megakaryocytic, granulocytic and monocytic differentiation and maturation. (*right*) Schematic representation of May-Grünwald-Giemsa staining of unilineage erythroid, megakaryocytic, granulocytic and monocytic cultures at various differentiation stages. Percentage of blasts, differentiating and mature cells with respect to total cells is indicated. Representative experiments are presented.

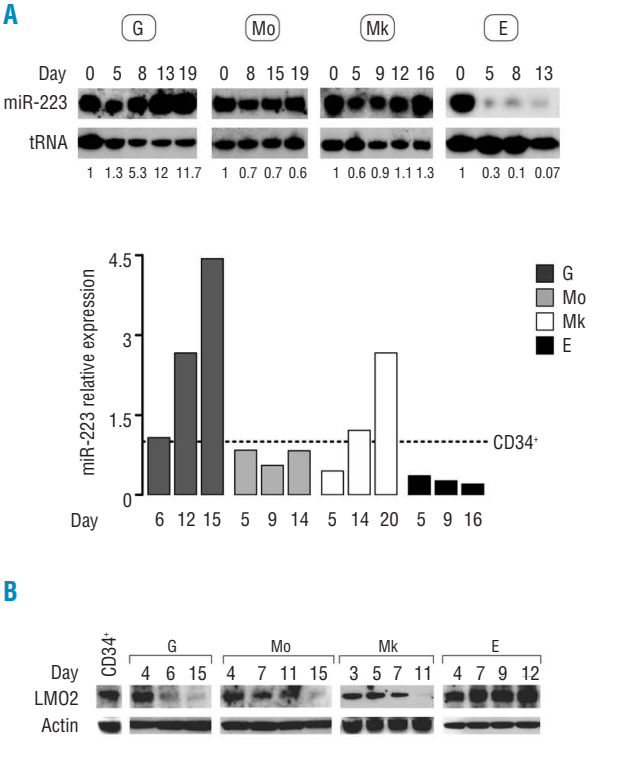

Figure 2. miR-223 expression is inversely related to LMO2 expression in hematopoietic cultures. (A) (*top*) Northern blot analysis of miR-223 expression performed on the indicated days in HPCs grown in liquid phase unilineage granulocytic, monocytic, megakaryocytic and erythroid cultures. Loading RNA control was performed with Met-tRNA. Values indicate the ratio between miR-223 and tRNA normalized on day 0 (CD 34⁺ cells). (*bottom*) qRT-PCR of miR-223 in G, Mo, Mk and E cultures. A representative experiment out of three is presented. (B) Western blot of LMO2 protein in CD34⁺ HPCs, granulocytic, monocytic, megakaryocytic and erythroid cultures. Actin was used as loading control. Values represented the ratio between LMO2 protein and actin normalized on day 0 (CD 34⁺ cells).

thus suggesting that this decrease may be a necessary event to allow the expression of relevant proteins involved in erythropoiesis.

LMO2 is a direct target for miR-223

Searching for proteins targeted by miR-223 and specifically involved in the erythroid differentiative process, we first used a computational and bioinformatics-based approach (TargetScan: *www.targetscan.org, and PicTar: www.pictar.bio.nyu.edu*) which predicted a number of putative targets. Among them, as a good candidate we focused on LMO2 for its essential role in erythropoiesis.[4-9] Initially, we analyzed the expression level of LMO2 in unilineage G, Mo, MK and E by Western blot (Figure 2B). LMO2 protein sharply decreases during granulocytic, monocytic and megakaryocytic differentiation being barely or not detectable in differentiated cells. On the contrary LMO2 is induced and highly expressed during erythropoiesis (Figure 2B). Thus, the expression of miR-223 and LMO2 are inversely related in each type of unilineage culture consistently with the hypothesis that LMO2 may be a direct target of miR-223.

In order to verify whether LMO2 mRNA is a direct target of miR-223, we cloned the 3'UTR of the LMO2 gene downstream of the renilla reporter gene (psiCHECKTM-2 vector). Bioinformatic analysis predicted that the seed sequence in miR-223 matches LMO2 3'UTR from 1743 to 1765 nucleotides (Figure 3A). The LMO2 3'UTR construct was co-transfected with either miR-223 oligonucleotides or non-targeting oligoribonucleotide (ctr-miRNA) into the HeLa cell line. This cell line is a useful system since it is easily transfectable and does not express miR-223 (*data not shown*). A 50% repression of the reporter activity was obtained with miR-223, while there was no reduction with the non-targeting miRNA (Figure 3A). Importantly, mutation of six bases (Figure 3A, top panel) in the candidate miR-223 binding site totally abrogated miR-223-dependent repression. These experiments demonstrate that miR-223 directly interacts with the LMO2 3'UTR.

miR-223 down-modulates LMO2 expression in erythroid cells

The effects of miR-223 overexpression in unilineage erythroid cultures of CB CD34$^+$ HPCs were evaluated.

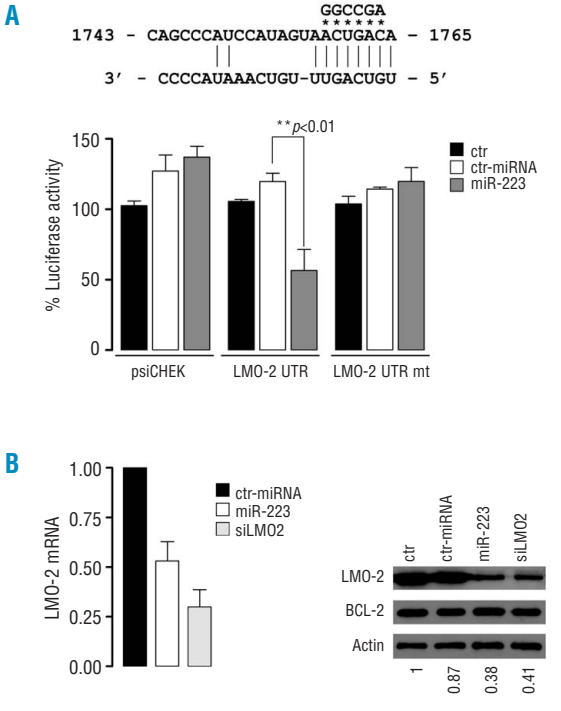

Figure 3. LMO2 is a target of miR-223. (A) (*top*) Putative miR-223 binding site in the LMO2 3'UTR. (*bottom*) Luciferase activity in HeLa cells co-transfected with either psiCHEK LMO2 3'UTR wild type and miR-223, or psiCHEK LMO2 3'UTR with mutated seed (mt) and miR-223. Controls were transfected with psi-CHEK LMO2 3'UTR or psiCHEK Promoter (empty vector), either in the absence of miRNA (ctr) or upon transfection of non-targeting oligonucleotide (ctr-miRNA). Renilla luciferase values normalized for firefly luciferase are presented. Mean + SD from four independent experiments. (B) (left) qRT-PCR of LMO2 mRNA expression. Error bars represent standard deviation and indicate the average values from three independent experiments. (*right*) Western blot analysis in unilineage E culture transfected on day 3 with miR-223, siLMO2 or control miRNA, compared with untreated E cells. BCL2 and actin were used as control. The values represented the ratio between LMO2 protein and actin normalized on control cells (ctr).

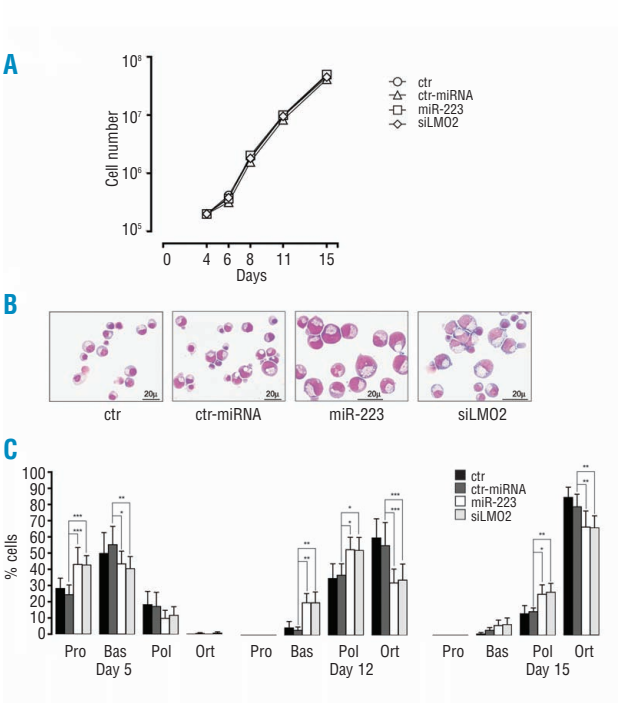

Figure 4. Transfection of miR-223 or siLMO2 does not affect cell growth but impairs E differentiation. (A) Growth curve of erythroid culture transfected with miR-223, siLMO2 or a non-targeting oligonucletide (ctr-miRNA) compared with untreated E cells. A representative experiment is shown. (B) Representative May-Grünwald-Giemsa staining analysis of erythroid cells nine days after transfection (day 12 of culture) with miR-223, siLMO2, control-miR compared with untreated E cultures (original magnification 400x for ctr-miRNA and untreated cells; original magnification 600x for miR-223, siLMO2 transfected cells). The size bars of 20 μ is included in each panel. (C) Percentage of proerythroblasts (Pro), basophilic (Bas), polychromatophilic (Pol) and orthocromatic (Ort) erythroblasts two days (day 5 of culture) (*left*) and thirteen days (day 15 of culture) (*right*) after transfection with miR-223, siLMO2, ctr-miRNA compared with untreated E cultures. The results are the mean + SD from five independent experiments.

CD34+ HPCs were grown in unilineage E liquid suspension culture and, as this miRNA declines during initial erythroid differentiation, cells were transfected on day 3 with miR-223 or the ctr-miRNA oligoribonucleotide. Small interfering RNA against LMO2 (siLMO2) was also utilized to confirm the role of LMO2 in erythropoiesis and to define the functional relevance of LMO2 targeting by miR-223 as related to E growth and differentiation. In order to eliminate any possible bias due to a low percentage of untransfected cells, a FITC conjugated double-stranded non-targeting RNA was also co-transfected with either miR-223 or ctr-miRNA. After transfection FITC-positive cells were sorted and cultured in erythroid medium. The analysis of LMO2 mRNA and protein expression in E transfected culture was performed two and three days after transfection respectively. As shown in Figure 3B (left) enforced miR-223 expression resulted in degradation of LMO2 mRNA, as well as treatment with siLMO2. Accordingly, LMO2 protein level was markedly lower in miR-223- or siLMO2 transfected cells, as compared with control groups (Figure 3B, right). The observed smaller decrease in LMO2 mRNA compared to LMO2 protein (Figure 3B) might be the result of miRNA-mediated mRNA-decay coupled to translational repression.[32] Furthermore, we reprobed the membrane of Western blot with either anti-actin antibody for loading control or anti-BCL2 antibody that is not predicted to be a target of miR-223, to assess miRNA specificity. As expected BCL2 protein level was not affected by miR-223 (Figure 3B right).

No differences in the proliferative rates were found when miR-223 and siLMO2 treated cells were compared with the control cells (Figure 4A).

The morphological analysis of erythroid cell differentiation showed a significant increase in the proportion of immature erythroblasts (proerythroblasts and basophilic erythroblasts) either in miR-223 or siLMO2 treated cells and a concomitant decrease in mature erythroblasts (orthochromatic), as evaluated at different stages of differentiation/maturation. This result demonstrates an inhibitory effect of miR-223 on erythroid maturation (Figure 4B,C).

To further confirm these observations erythroid cells at day one of culture were transfected with anti-miRNA oligonucleotide directed against miR-223. Anti-miRNAs are 2'-O methyl-antisense oligonucleotides that bind and irreversibly inactivate miRNAs specifically inhibiting miRNA function.[33] Anti-miRNA treated cells showed only a slight increase in cell differentiation when compared to control cells (*data not shown*). These results are in line with the expression profile of miR-223 showing a marked decline as early as day 5 of culture (Figure 2A).

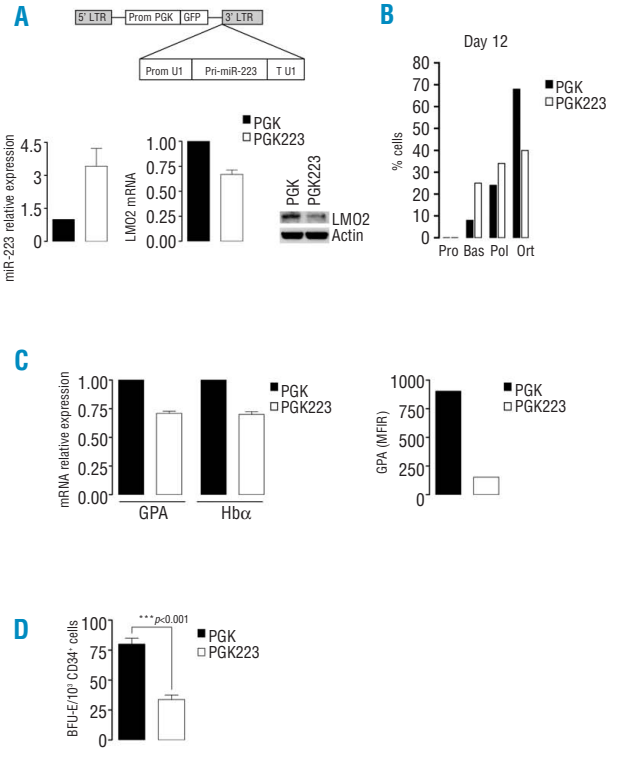

Figure 5. Ectopic miR-223 expression in E cells downregulates LMO2 protein expression and impairs erythroid differentiation, maturation and E progenitor clonogenesis. (A) (*top*) Schematic representation of the lentiviral vector for miR-223 expression (PGK223). (*bottom, left*) qRT-PCR of miR-223 expression in erythroid cells at day 3 of culture infected with the empty vector or with PGK-223. (*bottom, middle*) Analysis of LMO2 expression by qRT-PCR. Error bars represent standard deviation and indicate the average values from three independent experiments. (*bottom, right*) Western blot analysis in E cells at day 3 of culture infected with the empty vector or with PGK223. Values indicated the ratio between LMO2 protein and actin normalized on control cells (i.e. CD34+ HPCs transfected with the empty vector). (B) Percentage of differentiating erythroid cells infected with miR-223 or empty vector at day 12 of culture. A representative experiment out of three experiments is shown. (C) (*left*) Analysis of GPA and Hba mRNAs expression by qRT-PCR in E cells infected with the empty vector or with PGK223. The results are the mean±SD from three independent experiments. (*right*) Mean fluorescence intensity ratio (MFIR) of GPA in E cells transduced with PGK223 or empty vector analyzed six days post-infection. The values are reported as the ratio between mean fluorescence intensity of the sample and the isotype control. A representative experiment out of three is shown. (D) Erythroid colony-forming capacity of HPCs transduced with miR-223, as compared with cells transduced with empty vector. Mean±SD from three independent experiments.

Enforced expression of miR-223 in E cells impairs differentiation and erythroid colony formation

To further investigate the role of miR-223 during normal erythroid differentiation we transduced CB CD34+ HPCs with a lentiviral construct harboring miR-223 and the *GFP* gene[21] (Figure 5A). After transduction, the cells efficiently expressing GFP were selected by fluorescence-activated cell sorting (FACS) and grown in erythroid unilineage culture to drive their selective differentiation. After 48 h, the resulting expression level of miR-223 in infected cells (PGK223) was about three fold the amount measurable in cells infected with the empty vector (PGK) (Figure 5A). The analysis of LMO2 mRNA and protein expression in transduced E cultures was performed three days after transfection. As shown in Figure 5A the enforced expression of miR-223 resulted in degradation of LMO2 mRNA and in a decrease of about 60% of LMO2 protein levels.

As expected, morphological analysis of E cells over-expressing miR-223 showed a higher number of immature cells and a lower fraction of mature orthocromatic erythroblasts with respect to control cells (Figure 5B)

whereas both cell populations showed a similar cell proliferation rate (*data not shown*). To further confirm the functional relationship between miR-223 and the erythroid differentiation, we evaluated the levels of Glycophorin A (GPA), an erythroid-specific surface marker, and of the hemoglobin α chain (Hbα). qRT-PCR analysis showed a reduced expression of GPA and Hba mRNAs in miR-223 expressing cells compared with CD34$^+$ cells transduced with empty vector (Figure 5C, left). Next, we measured the expression level of Glycophorin A (GPA) by flow-cytometry. Consistently, this analysis showed a reduced protein expression at single cell level in miR-223 expressing cells compared with CD34$^+$ cells transduced with empty vector (Figure 5C, right).

These observations were confirmed by clonogenic studies on HPCs in methylcellulose medium. Fluorescence positive HPCs transduced with PGKmiR-223 or control vector were selected and plated (three days post-transduction) in semisolid E culture. After 11-12 days of culture the plates were scored to evaluate the number of BFU-E colonies. The erythrocytic clonogenetic capacity of HPCs expressing miR-223 was reduced to 40% of the control value (Figure 5D).

Discussion

MiRNAs play an important general role in the mechanism of cell differentiation and maintenance of cell identity, but relatively little is known of their functional role in modulating hematopoietic lineage differentiation.

Although an elaborate set of transcriptional regulatory mechanisms seems to be responsible for the differentiative process, a network of post-transcriptional regulation, miRNA-mediated, is also observed. Particularly, miR-223 has been demonstrated to regulate granulocytic differentiation inhibiting translation of specific transcription factors, but the role of miR-223 in the other lineages still had to be explored.

In this study, we analyzed miR-223 expression in serum-free culture systems for unilineage differentiation and maturation of cord blood (CB) CD34$^+$ hematopoietic progenitor cells through the erythroid, megakaryocytic, granulocytic and monocytic pathways.

Our study indicates that miR-223 is markedly down-modulated only during erythroid differentiation and maturation and this event is necessary to promote erythropoiesis at both progenitor and precursor levels. In fact, enforced expression of miR-223 in unilineage erythroid culture of CD34$^+$ progenitor cells inhibits both commitment of hematopoietic progenitor cells, as indicated by clonogenic studies, and differentiation of precursor cells, as documented by liquid phase culture experiments.

On the basis of bioinformatic analysis, we hypothesized that the effects of miR-223 on erythropoiesis may be mediated by LMO2 targeting, an essential gene for erythroid differentiation.[4-10] Our study indicates that in erythroid culture LMO2 expression is inversely related to the level of miR-223. Treatment of E cultures with miR-223 or LMO2 siRNA similarly down-regulates LMO2 protein and similarly impairs erythroid differentiation. These results indicate that miR-223 exerts its inhibitory role in erythroid cells through LMO2, although we cannot exclude the possibility that miR-223 may also target other genes relevant for erythropoiesis. This evidence is supported by computational analysis which indicate that miR-223 might also target FOXO3A[34,35] and Sp3,[36] two transcription factors involved in erythroid differentiation.

Moreover, bioinformatic analysis indicated that miR-223 is the only known miRNA predicted to bind to the LMO2 3'UTR. Therefore, the possibility that other miRNAs may co-operate with miR-223 to block erythropoiesis through the repression of LMO2 protein expression seems to be excluded. This is also suggested by the similar reduction of LMO2 mRNA obtained either by siLMO2 and by miR-223. Interestingly, miR-223[17] and LMO2[37,38] (*A. Zeuner, unpublished data, 2006*) expression are inversely related in erythroid cultures derived from adult blood suggesting that LMO2 regulation may be a relevant mechanism involved also in the control of adult erythropoiesis.

Several miRNAs participate in the regulation of erythropoiesis: miR-221/222 inhibit normal erythropoiesis and erythroleukemic cell growth down-regulating kit receptor expression,[28] miR-24 hampers erythropoiesis by targeting ALK4 and perturbing activin signaling,[39] miR-320 finely tunes the translational activities of CD71 in reticulocytes,[20] c-Myb and miR-15a form an autoregulatory feedback loop which govern the transition from BFU-E to CFU-E stage.[40]

Our results suggest that also miR-223 belongs to a functional set of miRNAs implicated in fine-tuning the expression of key genes to a physiologically relevant level during erythropoiesis.

Altogether, these findings suggest that the post-transcriptional regulation by miRNAs of hematopoietic transcription factors or growth factor receptors may play an important role in the mechanism of cell differentiation and maintenance of cell identity. In fact, the gene program underlying differentiation/maturation of the hematopoietic lineages involves regulatory circuitries mediated by a cohort of lineage(s)-specific miRNAs, targeting key functional genes at mRNA level.

Authorship and Disclosures

NF designed and performed research, analyzed and interpreted the data, assisted in writing the manuscript; FP performed experiments; PR performed experiments; MB performed experiments; OM performed morphological analysis; GC prepared unilineage cultures; SS prepared unilineage cultures; SC performed research; AS performed preliminary experiments and provided essential reagents; CP discussed the data; GM analyzed and interpreted the data, and wrote the paper.

The authors reported no potential conflicts of interest.

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