

MicroRNA-125b transforms myeloid cell lines by repressing multiple mRNA

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ABSTRACT

Background

We previously described a t(2;11)(p21;q23) chromosomal translocation found in patients with myelodysplasia or acute myeloid leukemia that leads to over-expression of the microRNA miR-125b, and we showed that transplantation of mice with murine stem/progenitor cells over-expressing miR-125b is able to induce leukemia. In this study, we investigated the mechanism of myeloid transformation by miR-125b.

Design and Methods

To investigate the consequences of miR-125b over-expression on myeloid differentiation, apoptosis and proliferation, we used the NB4 and HL60 human promyelocytic cell lines and the 32Dclone3 murine promyelocytic cell line. To test whether miR-125b is able to transform myeloid cells, we used the non-tumorigenic and interleukin-3-dependent 32Dclone3 cell line over-expressing miR-125b, in xenograft experiments in nude mice and in conditions of interleukin-3 deprivation. To identify new miR-125b targets, we compared, by RNA-sequencing, the transcriptome of cell lines that do or do not over-express miR-125b.

Results

We showed that miR-125b over-expression blocks apoptosis and myeloid differentiation and enhances proliferation in both species. More importantly, we demonstrated that miR-125b is able to transform the 32Dclone3 cell line by conferring growth independence from interleukin-3; xenograft experiments showed that these cells form tumors in nude mice. Using RNA-sequencing and quantitative real-time polymerase chain reaction experiments, we identified multiple miR-125b targets. We demonstrated that ABTB1, an anti-proliferative factor, is a new direct target of miR-125b and we confirmed that CBFβ, a transcription factor involved in hematopoiesis, is also targeted by miR-125b. MiR-125b controls apoptosis by down-regulating genes involved in the p53 pathway including *BAK1* and *TP53INP1*.

Conclusions

This study demonstrates that in a myeloid context, miR-125b is an oncomiR able to transform cell lines. miR-125b blocks myeloid differentiation in part by targeting CBFβ, blocks apoptosis through down-regulation of multiple genes involved in the p53 pathway, and confers a proliferative advantage to human and mouse myeloid cell lines in part by targeting ABTB1.

Key words: miR-125b, myeloid differentiation, apoptosis, proliferation, CBFβ, ABTB1.

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The online version of this article has a Supplementary Appendix.

Introduction

MicroRNA modulate a variety of cellular pathways, including development, differentiation, proliferation, and apoptosis, and dysregulation of microRNA expression underlies specific oncogenic events in human cancer.^{1,2}

In a previous study, we showed that a t(2;11)(p21;q23) chromosomal translocation found in 19 patients with acute myeloid leukemia or myelodysplastic syndrome leads to up-regulation of microRNA miR-125b compared to the levels in healthy individuals and leukemic patients lacking this translocation.³ MiR-125b is expressed at high levels in many other cancers. For example, miR-125b is over-expressed in Down syndrome patients with megakaryoblastic leukemia.⁴ Its over-expression is also found in association with several chromosomal translocations including *TEL-AML1* in acute lymphoid leukemia,⁵ *PML-RARA* in acute promyeloblastic leukemia⁶ and *BCR-ABL* in chronic myeloid leukemia and B-cell acute lymphoblastic leukemia.⁷ MiR-125b is also involved in the t(11;14)(q24;q32) chromosomal translocation found in B-cell acute lymphoblastic leukemia, which juxtaposes the immunoglobulin heavy chain enhancer to the miR-125b locus leading to miR-125b over-expression.⁸ In solid tumors, miR-125b is over-expressed in prostate⁹ and colorectal¹⁰ cancers. Interestingly, miR-125b was found to be down-regulated in breast^{11,12} and oral¹³ cancers, in melanoma¹⁴ and in hepatocellular¹⁵ and thyroid anaplastic carcinomas.¹⁶ Thus miR-125b seems to have a dual role depending on the cell type or context. It can act as an onco-microRNA (onco-miR) in hematologic malignancies by targeting tumor suppressor genes or as a tumor suppressor miR in breast cancer by targeting oncogenes. For example, miR-125b targets multiple genes involved in the p53 pathway and induces a blockage of apoptosis in human neuroblastoma cells.¹⁷ However, in breast cancer, in which it is down-regulated, miR-125b cannot regulate its targets, leading to over-expression of the *ETS1*¹¹ or *MUC1*¹⁸ oncogenes.

In vitro experiments showed that miR-125b over-expression blocks granulocytic and monocytic differentiation of human promyelocytic leukemic cell lines and perturbs myeloid differentiation of primary mouse cells.^{3,4} *In vivo*, transplantation experiments in mice by miR-125b-over-expressing lineage-negative cells perturb hematopoiesis and in some conditions induce hematologic malignancies.^{19,21} High miR-125b expression leads to the development of acute myeloid leukemia²⁰ and lower expression can induce B-cell or T-cell acute lymphoid leukemia in transplanted mice.¹⁹ Enomoto *et al.* developed a transgenic mice model mimicking the t(11;14)(q24;q32) chromosomal translocation found in patients with B-cell acute lymphoblastic leukemia; these mice over-expressed miR-125b driven by the *IGH* enhancer and promoter and developed lethal B-cell malignancies with clonal proliferation.⁷

Normally miR-125b is highly expressed in hematopoietic stem cells (HSC) and its expression decreases in committed progenitors.^{20,22} MiR-125b over-expression in HSC confers better engraftment in transplanted mice.^{20,22}

In this study, using human and mouse myeloid cell lines, we examined the role of miR-125b as an oncomiR in myeloid malignancies.

Design and Methods

Cell culture, transfection and transduction

NB4 and 32Dclone3 cell lines were purchased from the *Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH* (DSMZ) and American Type Culture Collection (ATCC), respectively. HL60E expressing the murine ecotropic receptor was a generous gift from Brad Fletcher. The 293T cell line was purchased from the ATCC.

NB4 cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum, L-glutamine, penicillin and streptomycin. Transient transfections of microRNA negative control #1 (Dharmacon CN-001000-01) or hsa-miR-125b mimic (Dharmacon C-300595-03-0005) (22.5 μ L of each mimic at the concentration of 50 μ M) into NB4 cells (3×10^6) were performed by electroporation at 200 V and 950 μ F, using Pulser (BioRad). Transient transfections of microRNA hairpin inhibitor negative control #1 (Dharmacon IN-001005-01) or mmu-miR-125b inhibitor (Dharmacon IH-310393-07) (8 μ L of each inhibitor at a concentration of 100 μ M) into NB4 or 32Dclone3 cells (3×10^6) were performed by electroporation at 200 V and 950 μ F, using Pulser (BioRad). HL60E were cultured in Iscove's modified Dulbecco's medium (Gibco) supplemented with 1.5 g/L sodium bicarbonate and 10% fetal bovine serum. 32Dclone3 cells were grown in 10% fetal bovine serum, 10% interleukin-3 (IL-3) (WEHI media) and 1% penicillin and streptomycin (Gibco). 32Dclone3 and HL60E were stably infected with XZ or XZ-miR-125b, as described previously.¹⁹ Infection was performed twice with two different virus supernatants for each condition. Then, all of the experiments were performed at least twice for each of the infected cells.

Differentiation assay

Differentiation of 32Dclone3 was induced by adding granulocyte colony-stimulating factor at a final concentration of 100 ng/mL to the media. Five days later, cells were stained with anti-CD11b and anti-Gr1 antibody for fluorescence activated cell sorting (FACS) analysis on an LSRII (BD Biosciences). Morphological analysis was performed with May-Grünwald Giemsa staining (Sigma Aldrich) and slides were visualized under a AxioCam MRC microscope (Zeiss).

Apoptosis assay

For NB4 and HL60E, apoptosis was induced with camptothecin at a final concentration of 10 μ M. The cells were harvested at day 2 after induction and stained using an annexin V-phycoerythrin/7-aminoactinomycin apoptosis detection kit (BD Biosciences) according to the manufacturer's instructions. For 32Dclone3, apoptosis was triggered by removing IL-3 from the media and stained 4 days later for flow cytometry analysis.

Proliferation assay

32Dclone3 and HL60E infected cells (green fluorescent protein-positive; GFP⁺) were mixed with wild-type cells (green fluorescent protein-negative; GFP⁻) at a ratio of 1:3. The percent of GFP⁺ cells was determined by FACS analysis every 3 days for 15 days.

For cell cycle analysis, cells were collected, washed, suspended in cold phosphate-buffered saline, fixed in 80% ethanol and stained with propidium iodide.

mRNA-sequencing

For each cell line, four samples were used to generate four libraries, which were duplicates of two conditions: 32Dclone3 control (1 and 2) and 32Dclone3 125b (1 and 2); NB4 transiently transfected with control mimics (1 and 2) and NB4 transiently transfected with miR-125b mimics (1 and 2), at day 3 post-transfection. Total RNA of the cells was extracted using a RNeasy kit

(Qiagen) and mRNA libraries of each sample were prepared following instructions and using reagents from Illumina/Solexa. The libraries were prepared using polyA+ enriched RNA according to the manufacturer's instructions (Illumina) and then sequenced on a Solexa sequencing cell. All reads were aligned to the mouse mm9 genome or to the human genome (HG18) using the UCSC database for 32Dclone3 and NB4, respectively. Gene expression values were calculated as Reads Per Kilobase of Exon Model Per Million Mapped Reads (RPKM). The expression threshold of 1 RPKM was used as a cut-off for sets of aligned genes. The RPKM values from miR-125b over-expressing cells were expressed as a fold-change relative to control cells. The RNA-seq analysis data have been deposited at the National Center for Biotechnology Information Gene Expression Omnibus (repository numbers GSE37018 and GSE37061). To calculate the enrichment of miR-125b targets among the genes down-regulated in both cell lines, we used the software available at http://serge.mehl.free.fr/anx/loi_hypergeo.html. Population size: 10417 for 32Dclone3 and 10772 for NB4; sample size: 83; number of events with the selected criteria: 1244; success in the sample: 25

Quantitative real-time reverse transcriptase polymerase chain reaction analysis

Total RNA was isolated using the Trizol extraction protocol according to the manufacturer's instructions. Reverse transcription was performed with Superscript II reverse transcriptase (Invitrogen). Quantitative polymerase chain reaction (PCR) was performed with SYBRgreen Master Mix (Applied Biosystems) on 96-well plates using ABI 7600 and the primers listed in *Online Supplementary Table S1*. The data presented correspond to the mean of 2^{ΔΔCt} from at least three independent experiments, normalized to the mouse *Gapdh* and/or *Hprt* reference genes for 32Dclone3 experiments and to the human *MLN51* and *ACTIN* reference genes for NB4 and HL60 experiments.

Western blot

The cells were harvested and lysed by ELB buffer [250 mM NaCl, 0.1% Nonidet P-40, 50 mM HEPES (pH 7.0), 5 mM EDTA] containing proteinase inhibitor cocktail (Roche). For western blotting, 50 μg of protein were denatured by NuPAGE loading buffer (Invitrogen) at 70 °C for 10 min. Western blots were probed with the following rabbit polyclonal antibodies: anti-CBFB (ab33516, Abcam), anti-ABTB1 (ab99547, Abcam) and anti-GAPDH (sc-25778, Santa Cruz Biotechnology).

Luciferase assay

Using primers listed in *Online Supplementary Table S1*, fragments corresponding to 3'UTR of putative targets containing the binding sites for miR-125b were amplified by PCR. The PCR products were cloned into reporter vector pscheck2 (Promega) using XhoI and NotI restriction sites.

Primers reported in *Online Supplementary Table S1* were used for site-directed mutagenesis. The mutagenesis PCR reaction was performed with 50 ng of plasmid, 1 μL of primers (10 μM each), 1 μL dNTP mixture (10 mM each) and PfuTurbo DNA polymerase at the concentration of 2.5 U/μL in a thermal cycler (BioRad) at 95 °C for 30 s, then 22 cycles of 95 °C for 30 s, 53 °C for 1 min and 66 °C for 8 min. DpnI was added to digest the non-mutated parental DNA templates.

One day before transfection, 293T cells were seeded into 96-well white plates at 1-2x10⁴ cell/well. Cells were co-transfected with 1 μL of 1 μM miR-125b mimic or control mimic (Dharmacon) and 10 ng of constructs by using Lipofectamin 2000 according to the manufacturer's protocol (Invitrogen). The luciferase activity was measured 48 h after transfection using a

Dual-Glo Luciferase kit (Promega) and TECAN luminescence reader. The *Renilla* luciferase signal, which accounts for the effect of miR-125b on the 3'UTR of the *Renilla* gene, was normalized to the Firefly luciferase signal, which is an internal control.

Xenograft experiments in nude mice

All experimental protocols were approved by the Ethics Committee for Animal Experimentation of the Whitehead Institute for Biomedical Research. In these experiments 1x10⁷ cells of 32Dclone3 XZ-miR-125b [1 and 2 (10 mice each)], 32Dclone3 XZ [1 and 2 (10 mice each)] or 32Dclone3 cells independent of IL-3 [1 and 2 (5 mice each)] were injected subcutaneously into nude mice (Taconic CrTac:NCr-Foxn1tm). The endpoint was the time when the cells formed an aggregate tumor greater than 1 cm in diameter. Euthanasia was achieved by CO₂ inhalation.

Results

miR-125b over-expression blocks differentiation and apoptosis and induces proliferation of human and mouse myeloid cell lines

32Dclone3 is a mouse promyelocytic cell line that can be induced toward granulocytic differentiation by addition of granulocyte colony-stimulating factor. This cell line is also dependent on IL-3 and so is a good model for studying apoptosis induced by cytokine deprivation. We previously reported that transient transfection of miR-125b mimics into the human promyelocytic leukemic cell lines NB4 and HL60 blocks granulocytic and monocytic differentiation in the presence of all-*trans* retinoic acid and dimethylsulfoxide, respectively.³ HL60E cells express the murine ecotropic receptor and allowed us to use the bicistronic murine retroviral vector (XZ) system to stably induce expression of mature miR-125b; the vector contains a RNA polymerase II promoter driving miRNA expression followed by an internal ribosome entry site and GFP.¹⁹ Thus GFP expression marks cells ectopically expressing miR-125b.

HL60E and 32Dclone3 cell lines were transduced with XZ-miR-125b or the empty vector XZ alone, sorted for GFP⁺ cells, and tested for apoptosis, proliferation and differentiation. In parallel the NB4 cell line was transiently transfected with miR-125b mimic or a negative control mimic.

Quantitative reverse transcriptase PCR experiments showed a miR-125b over-expression of approximately 4000-fold in transiently transfected NB4 cells, 3000-fold in infected HL60 cells and 2000-fold in infected 32Dclone3 cells compared to control cells (*Online Supplementary Figure S1B-D*). MiR-125b levels in patients with myeloid leukemia described in the literature range from 4-fold to 760-fold higher than those in the control samples (*Online Supplementary Figure S1A*).^{3,4,6,7,23} However, the level of expression of miR-125b in human samples was evaluated on total bone marrow cells, so miR-125b over-expression in leukemic blasts is largely underestimated. Of note, NB4 is a promyelocytic leukemic cell line with the *PML-RARA* chromosomal translocation but the endogenous level of miR-125b in NB4 is low and does not mimic the miR-125b over-expression found in human acute promyelocytic leukemia.²⁴ The main abnormality found in the HL60 human promyelocytic leukemic cell line is a c-Myc amplification and miR-125b is not highly expressed in these

cells.^{25,26} 32Dclone3 is a non-tumorigenic cell line derived from normal murine bone marrow and, like NB4, does not express a high level of endogenous miR-125b.²⁷ We used these cell lines because they are good models for studying *in vitro* apoptosis, proliferation and myeloid differentiation as they can be induced to differentiate after treatment.

Apoptosis was induced upon camptothecin treatment in NB4 and HL60E cells and by removal of IL-3 in 32Dclone3 cells. As shown in Figure 1A, 80% of control 32Dclone3 cells are apoptotic (annexin V⁺/7-aminoactinomycin D⁻) 4 days after removal of IL-3 compared to 30% of the cells over-expressing miR-125b. There was a similar relative decrease in apoptosis of NB4 and HL60E cells ectopically expressing miR-125b compared to control cells (Figures 1B-C). Thus miR-125b blocks apoptosis in both human and mouse cell lines. As previously observed with NB4 and HL60,³ 32Dclone3 cells ectopically expressing miR-125b show a blockage of granulocyte colony-stimulating factor-induced myeloid differentiation, as quantified both by FACS using the CD11b marker for granulocyte differentiation (*Online Supplementary Figure S2A*) and by morphological analysis (*Online Supplementary Figure S2B*).

To test the effects of miR-125b expression on cell growth, GFP⁺ HL60E or 32Dclone3 cells expressing miR-125b or the control vector were mixed with wild-type cells (GFP⁻) at a ratio of 1:3. As judged by the absence of changes of the ratio of GFP⁺ to GFP⁻ cells over time, the

control vector had no effect on the relative rate of division of either cell line. In contrast, the ratio of GFP⁺ miR-125b expressing cells to GFP⁻ control cells increases steadily over time, reflecting the proliferative advantage conferred by miR-125b over-expression. The doubling time of control 32Dclone3 cells was 19.1 h whereas that of miR-125b-expressing 32Dclone3 cells was 18 h (Figure 2A). In the HL60E cell line, the effect was even more dramatic with a doubling time of 34.3 h for control cells and 28.36 h for miR-125b-expressing cells (Figure 2B). By cell cycle analysis, we observed a proliferative advantage in miR-125b infected cells as shown by a decrease in the percentage of cells in the G1 phase and an increase in the percentage of cells in S phase (*Online Supplementary Figure S3*). Thus miR-125b confers a proliferative advantage in both human and mouse cell lines.

In summary, we demonstrated that miR-125b blocks differentiation, apoptosis and induces proliferation in mouse and human cell lines.

miR-125b is able to transform the 32Dclone3 cell line

To test whether miR-125b is able to transform myeloid cells, we used the mouse promyelocytic 32Dclone3 cell line, which is dependent on IL-3 for growth. In the absence of IL-3, all control 32Dclone3 cells died within 16 days. However, miR-125b 32Dclone3 cells survived and became independent of IL-3 for their growth (Figure 3A).

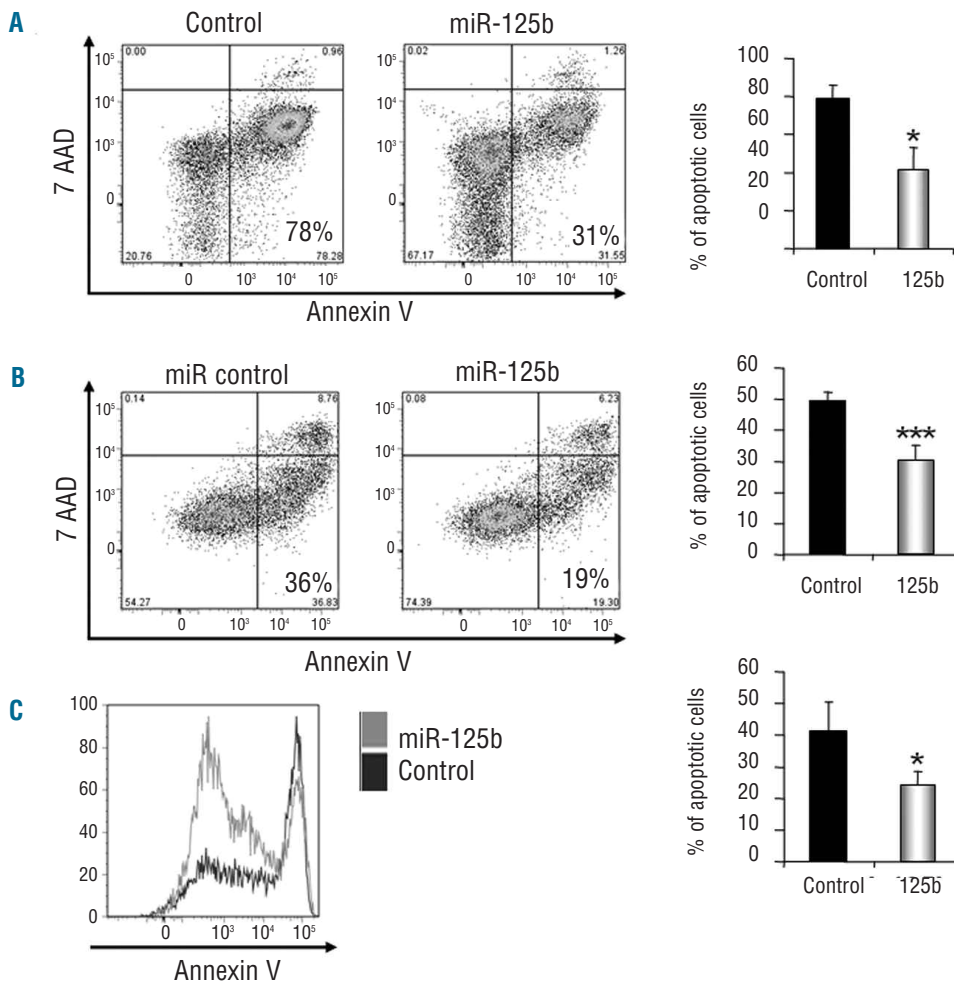


Figure 1. miR-125b blocks apoptosis of mouse and human cell lines (A) 32Dclone3 infected cells were deprived of IL-3 from the media and annexinV-phycoerythrin/7-aminoactinomycin D (7-AAD) staining was performed 4 days later. Annexin V⁺/7-AAD⁻ cells are apoptotic cells. One representative flow cytometry plot is shown. The histogram represents the average of apoptotic cells (annexin V⁺) from three independent experiments. (B) The human promyelocytic NB4 cell line was transiently transfected with a miR-125b mimic or mimic control. One day later, apoptosis was induced by adding camptothecin and the percentage of apoptotic cells was assessed 2 days later by annexin V/7-AAD staining. One representative flow cytometry plot is shown. The histogram represents the average of apoptotic cells (annexin V⁺) from five independent experiments. (C) The human promyelocytic HL60E cell line was stably infected with XZ or XZ-miR-125b. GFP⁺ cells were sorted and induced to apoptosis by camptothecin treatment. Apoptosis was quantified 2 days later by flow cytometry with annexin V staining. One representative flow cytometry plot is shown. The histogram represents the average of apoptotic cells (annexin V⁺) from three independent experiments.

Of note, some miR-125b over-expressing cells died at the beginning of the IL-3 deprivation as shown by 25% of apoptotic cells at day 2 after removal of IL-3 and then they recovered and grew in the absence of IL-3.

To evaluate the effect of miR-125b over-expression in tumor induction we used a 32Dclone3 xenograft model in nude mice. In this experiment 1×10^7 32Dclone3 cells over-expressing miR-125b or not were injected subcutaneously into the dorsal side of nude mice. MiR-125b 32Dclone3 cells produced an aggregate tumor burden greater than 1 cm in diameter within 65 to 75 days in all subcutaneously injected nude mice but no tumors were observed following injection with control 32Dclone3 cells (Figure 3B). When the tumor reached 1 cm of diameter the mice were sacrificed and analyzed for metastases. All of the mice injected with miR-125b over-expressing cells had splenomegaly, hepatomegaly, and huge lymph nodes full of miR-125b over-expressing cells (Figure 3C). In summary, miR-125b is an oncomiR able to transform the 32Dclone3 cell line.

Identification of miR-125b targets in myeloid cell lines

To better understand the mechanisms of blockage of myeloid differentiation and apoptosis and induction of

proliferation by miR-125b, we proceeded to identify miR-125b target genes involved in these pathways. We first analyzed the total cellular gene expression pattern by RNA-sequencing of the parental 32Dclone3 myeloid cell line and that ectopically expressing miR-125b. We generated four cDNA libraries corresponding to duplicates of miR-125b and control cells. The same experiment was done with the NB4 cell line transiently transfected with miR-125b or control mimics. The list of genes with decreased mRNA levels in the presence of miR-125b was then overlapped with the list of genes containing at least one predicted binding site in its 3'UTR matching the seed region of miR-125. A total of 2396 genes, irrespective of site conservation, are putative miR-125b targets in mice and 2964 genes in humans; 1244 genes are predicted targets in both mice and humans. As *Bak1* (Bcl-2 antagonist killer 1) and *PPP1CA* were previously described as miR-125b targets^{6,9,28} and were found to be down-regulated 1.24-fold/1.61-fold and 1.18-fold/1.34-fold, respectively, in duplicate experiments in miR-125b 32Dclone3 cells, we decided to use 1.15 as a threshold to identify down-regulated genes. For each cell line, genes down-regulated more than 1.15-fold in duplicate RNA-sequencing experiments were selected. For 32Dclone3 cells infected with XZ-miR-125b, 1366 genes out of 10417 expressed genes were down-regulated more than 1.15-fold compared to control cells infected with XZ in duplicate RNA-sequencing experiments (Online Supplementary Figure S4A). For NB4 cells transiently transfected with miR-125b mimics, 1272 genes out of 10772 expressed genes were down-regulated more than 1.15-fold compared to control cells transiently transfected with control mimics. Eighty-three genes were down-regulated in both 32Dclone3 and NB4 cells over-expressing miR-125b. Among these, 25 genes (Online Supplementary Table S2) are predicted miR-125b targets; hypergeometric analysis showed that there is an enrichment in predicted miR-125b targets among the genes down-regulated in both cell lines ($*P=5 \times 10^{-6}$).

Online Supplementary Figure S4B shows that the levels of mRNA bearing different predicted miR-125b binding sites - 8-mer, 7mer-m8, and 7mer-1A (for definitions, see <http://www.targetscons.org>) as defined by TargetScan, were indeed preferentially down-regulated in 32Dclone3 cells stably expressing miR-125b, compared to control mRNA that did not bear seed matches (black line). These cumulative curves validate our RNA-sequencing approach.

The 25 genes down-regulated in both cell lines and containing a predicted miR-125b binding site included *CBFB* (core binding factor beta), coding for a protein that plays crucial roles in hematopoiesis, especially in myeloid differentiation. *CBFB* was recently reported to be a miR-125b target in the human NB4 cell line.²⁹ To evaluate whether *CBFB* was a common direct target in both mouse and human cells, we quantified *CBFB* mRNA and protein levels by reverse transcriptase PCR and western blots in miR-125b 32Dclone3 cells and compared these levels to those in control cells. We did indeed observe down-regulation of mRNA and protein levels in miR-125b over-expressing cells (Online Supplementary Figure S5A-B). Similarly, application of an miR-125b inhibitor resulted in an increase in the level of *CBFB* protein (Online Supplementary Figure S5B). A luciferase assay demonstrated that *CBFB* was also a direct target of miR-125b in mouse (Online Supplementary Figure S5C). The observed suppression of reporter activity was completely disabled when only two nucleotides in

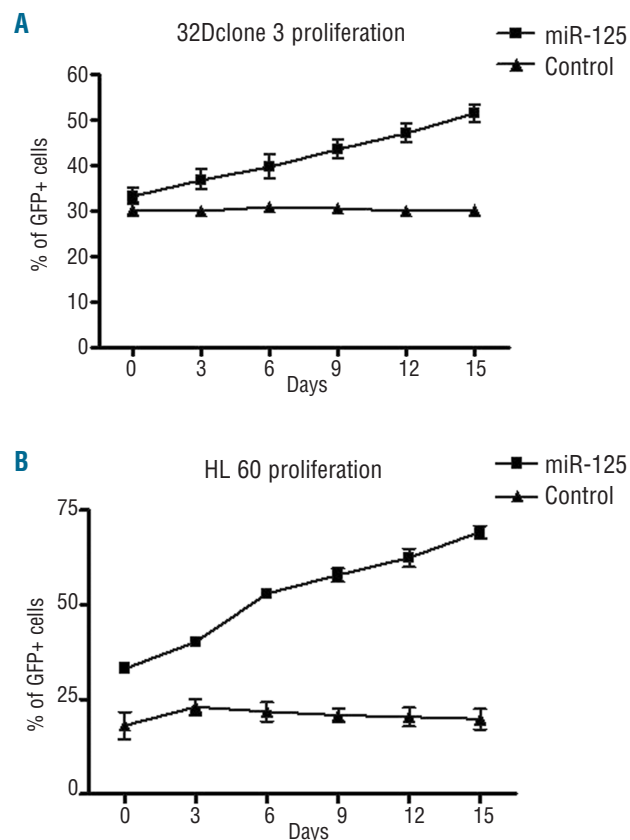


Figure 2. miR-125b confers a proliferative advantage to human and mouse myeloid cells. (A) 32Dclone3 infected cells (GFP⁺) expressing or not miR-125b were mixed with 32Dclone3 wild-type cells (GFP⁻) at a ratio ~1:3. The percent of GFP⁺ cells in the population was determined every 3 days of culture. Data represent the average of four independent experiments done with two different batches of virus for each condition. (B) The same experiment was performed with HL60E infected cells.

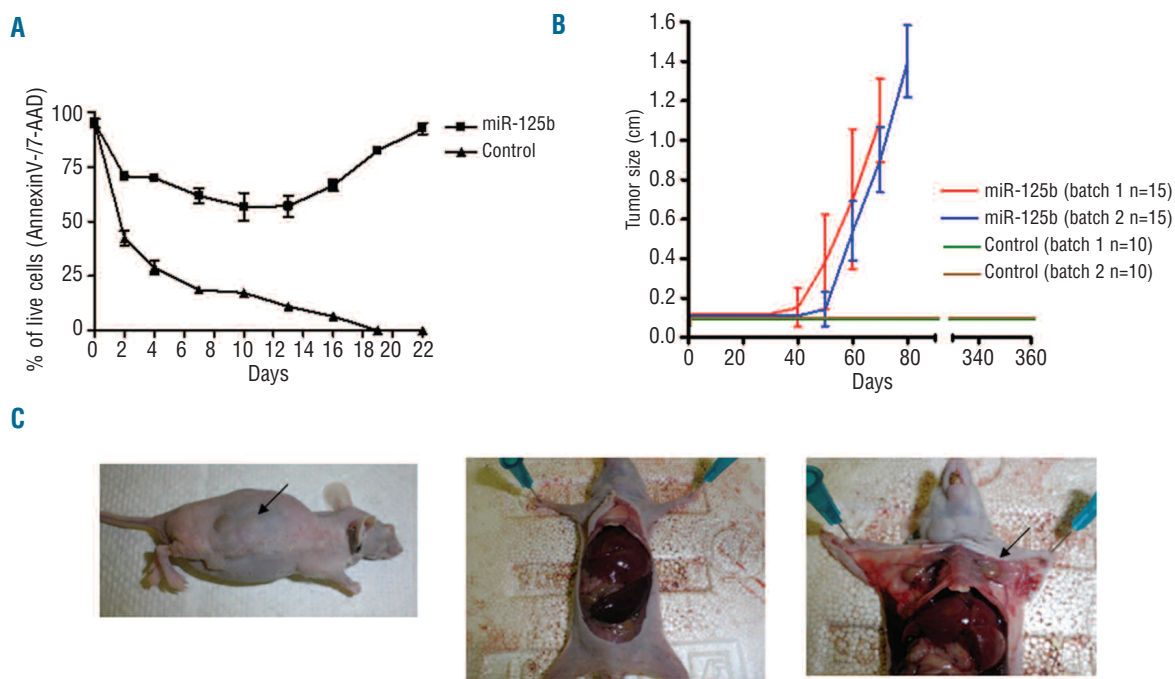


Figure 3. miR-125b is an oncomiR able to transform 32Dclone3 cells by conferring independence to growth factor removal and inducing tumors in nude mice (A) Independence from growth factors was assessed by removal of IL-3 from the 32Dclone3 media. Cells were resuspended at 300 000 cells/mL every 3 days. Viability of the cells was determined by flow cytometry analysis with annexin V/7-aminoadenine D (7-AAD) staining every 2 or 3 days. Annexin V⁺/7-AAD⁻ cells are live cells. Data represent the average of three independent experiments. (B) 1×10^7 32Dclone3 cells over-expressing miR-125b (n=30 total) or control (n=20 total) 32Dclone3 cells were subcutaneously injected into the backs of nude mice. Xenograft engraftment was monitored weekly and tumor size was recorded. Mice were sacrificed when the tumor reached 1 cm in diameter. Experiments were performed twice with 15 and 10 mice respectively injected with miR-125b overexpressing cells or control 32Dclone3 cells. (C) Pictures of nude mice injected with miR-125b 32Dclone3 cells showing the tumor (left), hepatomegaly and splenomegaly (middle) and infiltrated lymph nodes (right).

the putative microRNA responsive element were mutated, indicating that CBFβ is a direct target of miR-125b in mouse (*Online Supplementary Figure S5C*).

CBFβ plays a crucial role in hematopoiesis and it is highly expressed in hematopoietic stem cells, early stage myeloid lineage progenitors, and mature myeloid cells.³⁰⁻³² We postulated that deregulation of CBFβ expression by miR-125b could be the principal event in the blockage of myeloid differentiation following miR-125b overexpression in 32Dclone3 cells. Thus we used a short-hairpin (sh) RNA to knock-down CBFβ expression in 32Dclone3 cells. As shown in *Online Supplementary Figures S5D and S5E* by flow cytometry and morphology, respectively, down-regulation of CBFβ partially mimics the blockage of myeloid differentiation observed with miR-125b overexpression.

As miR-125b over-expression confers a proliferative advantage in mouse and human cells, we focused on the putative miR-125b target ABTB1 (*Online Supplementary Table S2*). ABTB1 is a tumor suppressor and mediator of the PTEN signaling pathway;³³ its over-expression in a colon cancer cell line leads to a decrease in proliferation and its knockdown confers a proliferative advantage to the cells.³³ At the mRNA level, *ABTB1* is down-regulated 3.9-fold in miR-125b 32Dclone3 cells compared to control cells (Figure 4A). There was a 60% decrease in ABTB1 protein expression in miR-125b overexpressing cells and a 40% increase in cells treated with a miR-125b inhibitor (Figure 4B). We validated ABTB1 as a direct miR-125b target by the luciferase assay shown in Figure 4C. However,

we were not able to observe an increase in proliferation of 32Dclone3 cells expressing an shRNA against ABTB1 (*data not shown*), likely because the level of ABTB1 protein was only partially reduced (Figure 4B).

We showed that miR-125b over-expression in human and mouse myeloid cell lines blocks apoptosis, but no pro-apoptotic genes were among the 25 genes found by RNA-sequencing (*Online Supplementary Table S2*). In Figure 4D we used the more quantitative reverse transcriptase PCR technique to assess the levels of several mRNA encoding pro-apoptotic proteins that other researchers had shown are direct miR-125b targets in various cells and species. As shown in Figure 4D, BAK1 and TP53INP1 were down-regulated by miR-125b over-expression in all three cell lines. Several pro-apoptotic genes, including *PLK3*, *PPP1CA* and *PRKRA*, were down-regulated in two of the three cell lines, while *PPP2CA* was significantly down-regulated only in HL60 cells. Thus miR-125b expression inhibits apoptosis by down-modulating levels of different pro-apoptotic genes in different myeloid cell lines.

Discussion

The microRNA miR-125b is often up-regulated in cancer, in particular in myeloid malignancies, and over-expression of miR-125b in transplanted murine stem/progenitor cells is able to induce leukemia. This study makes several novel points concerning the effects of miR-125b over-expression on myeloid progenitor cells. We showed

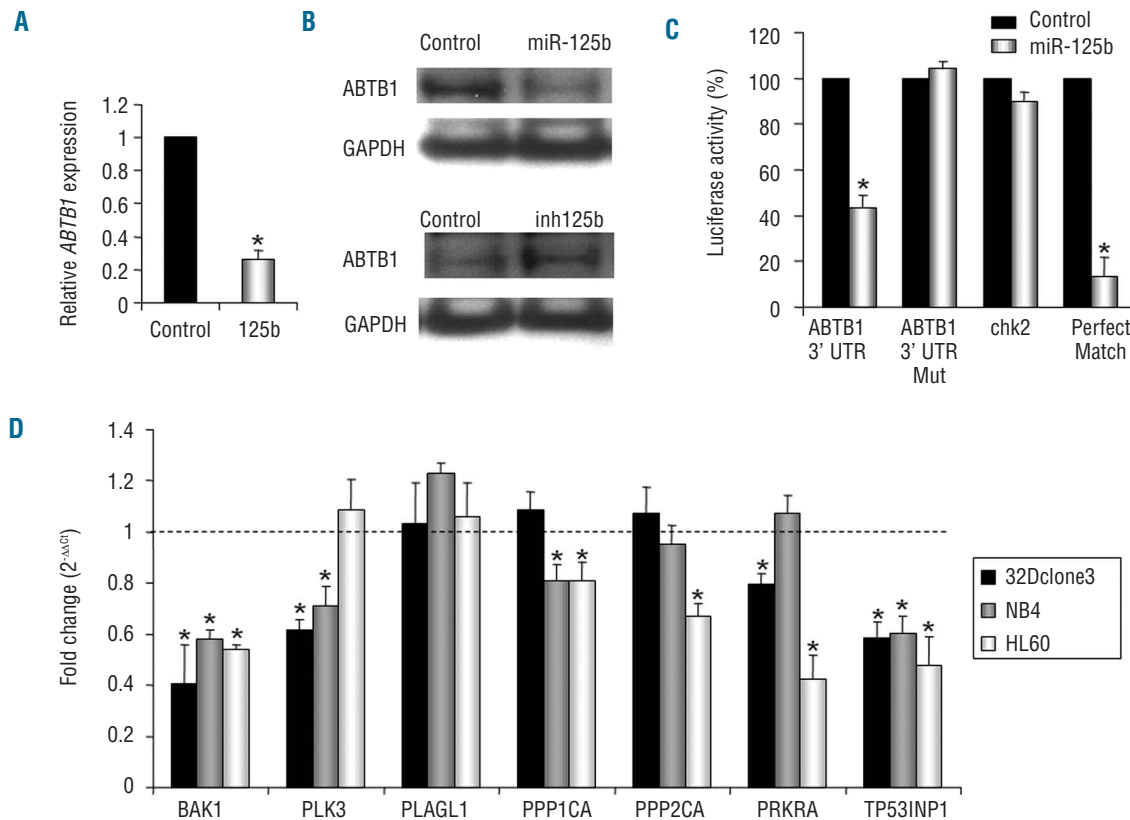


Figure 4. Identification of miR-125b targets: *ABTB1* is a miR-125b target. (A) Quantitative reverse transcriptase-PCR of *ABTB1* mRNA in 32Dclone3 cells overexpressing miR-125b compared to 32Dclone3 control. * $P < 0.0005$. (B) Western blot showing the down-regulation of *ABTB1* protein in miR-125b over-expressing cells compared to control cells (upper panel). *ABTB1* is increased in 32Dclone3 cells transiently transfected with an inhibitor of miR-125b (lower panel). (C) Repression of luciferase activity due to the binding of miR-125b to the 3'UTR of *ABTB1*. The 3'UTR of *ABTB1* containing the predicted binding site for miR-125b was cloned 3' to the *renilla* luciferase open reading frame in the psicheck2 vector. The *ABTB1* 3'UTR mut corresponds to the same construct with an internal mutation in the binding site of miR-125b. *chk2* is the empty vector and it serves as the negative control. The perfect match construct is the positive control containing the miR-125b binding site only. Each construct was co-transfected in 293T cells with miR-125b mimics or control mimics and luciferase activity was assessed 2 days after transfection. *Renilla* activity was normalized to the *firefly* internal psicheck control. The results presented correspond to the relative luciferase activity normalized to transfections with control mimics. * $P < 0.0005$. (D) miR-125b targets genes involved in apoptosis are down-regulated by miR-125b expression in both human and mouse myeloid cell lines. The mRNA expression levels of *BAK1*, *PLAGL1*, *PLK3*, *PPP1CA*, *PPP2CA* and *TP53INP1* were measured in 32Dclone3 and HL60 cells infected with XZ (control) or XZ-miR-125b vectors, and in NB4 cells 3 days after transient transfection with control or miR-125b mimics. The mRNA expression levels were evaluated by quantitative real-time PCR, normalized to the expressions of *MLN51* and *ACTIN* in human cells and *GAPDH* in mouse cells, and presented as fold change $[2^{-\Delta\Delta Ct}] \pm SD$ ($n \geq 3$) in miR-125b expressing cells relative to control cells. Two-tailed t-test results of * $P < 0.05$ relative to control cells.

that miR-125b over-expression is able to block myeloid differentiation, prevent apoptosis, and support cytokine-independent proliferation of both mouse and human myeloid cell lines. Furthermore, we showed that miR-125b expression is able to transform the 32Dclone3 cell line by making it independent of IL-3 for its growth and allowing it to form tumors in nude mice. We demonstrated that *ABTB1*, an anti-proliferative factor, is a new direct target of miR-125b, and showed that in these lines miR-125b also down-regulates a series of targets previously identified in other cell types or species, including CBFB, a transcription factor involved in hematopoiesis, as well as other genes upstream or downstream of p53 including *BAK1*, *TP53INP1*, *PLK3*, *PPP1CA*, *PRKRA* and *PPP2CA*. miR-125b expression promotes myeloid transformation by down-modulating levels of multiple genes that differ in different myeloid cell lines.

To identify miR-125b targets involved in myeloid differ-

entiation, apoptosis, and proliferation, we analyzed the total cellular gene expression pattern by RNA sequencing, comparing 32Dclone3 and NB4 cells over-expressing or not miR-125b. Among the down-regulated genes, we focused on those containing a predicted binding site for miR-125b in their 3'UTR. In different cell types miR-125b mediates its proliferative effects through down-regulation of several mRNA targets including *p53*,³⁴ pro-apoptotic Bcl-2 antagonist killer 1 (*bak1*),¹² Bcl-2 modifying factor (*bmf*),³⁵ and *TP53INP1*.³⁶ We identified a new putative miR-125b target involved in proliferation: *ABTB1* (Ankyrin repeat and BTB/POZ domain containing 1, also called *BPOZ*). Unoki *et al.* demonstrated that over-expression of *ABTB1* in the SW480 cell line decreased the rate of growth while suppression of *ABTB1* expression using anti-sense oligonucleotides resulted in an increased number of cells.³³ By using a luciferase reporter assay, we showed that *ABTB1* was a direct target of miR-125b. However, we

were not able to observe an increase in proliferation by using shRNA against *ABTB1* in 32Dclone3 cells, probably because of the low efficiency of shRNA knockdown of *ABTB1* compared to that induced by miR-125b over-expression.

Another important direct target of miR-125b is *CBFB*, which plays crucial roles in hematopoiesis, especially in myeloid differentiation. *CBFB* associated with *AML1* forms the core binding factor complex.³⁷⁻³⁹ *AML1* binds directly to the enhancer DNA sequence of target genes and *CBFB* increases the affinity and stabilizes the binding of *AML1* to DNA.^{37,39} *CBFB* and *AML1* are commonly deregulated in acute myeloid leukemia and form part of chimeric genes that can trigger cancer.⁴⁰ *CBFB* is involved in acute myeloid leukemia; the inversion of chromosome 16 [inv(16)(p13q22)] and the t(16;16)(p13;q22) chromosomal translocation both lead to the formation of an oncogenic fusion protein *CBFB-MYH11* (muscle myosin heavy chain 11).^{41,42} The oncogenic mechanism of *CBFB-MYH11* remains to be elucidated. Nonetheless, mice lacking the *Cbfb* gene or heterozygous for a *Cbfb/MYH11* allele produce an identical phenotype in which the animals undergo early embryonic death in part caused by the lack of fetal liver hematopoiesis.^{43,44}

Surdziel *et al.* found another miR-125b target involved in myeloid differentiation, the transcription factor *STAT3*.²⁸ They showed, by luciferase assays and western blotting, that *STAT3* was a direct target of miR-125b and that a strong reduction of *STAT3* expression by shRNA blocks granulocytic differentiation of 32Dclone3 cells.²⁸

MicroRNA down-regulate multiple mRNA targets that differ in different cell types and species; they should be considered more as fine regulators of networks than strong regulators of a single gene. We thus hypothesize that the blockage in myeloid differentiation mediated by miR-125b over-expression is due to partial down-regulation of a combination of genes including *CBFB*, *STAT3* and *ABTB1*. Similarly, the ability of miR-125b to block apoptosis in different cell types in different vertebrate species is due to its ability to partially down-regulate sets of proapoptotic genes in the *p53* network, but few specific genes, are conserved as miR-125b targets. For example, *p53* is a *bona fide* miR-125b target in humans and zebrafish

but not in mice.¹⁷ Other miR-125b target genes in the *p53* network include apoptosis regulators such as *Bak1*, *Igfbp3*, *Itch*, *Puma*, *Prkra*, *Tp53inp1*, *Tp53*, *Zac1*, and cell-cycle regulators including *cyclin C*, *Cdc25c*, *Cdkn2c*, *Edn1*, *Ppp1ca*, and *Sel1l*.³⁴ We showed that two of these genes, *BAK1* and *TP53INP1*, were down-regulated by miR-125b over-expression in all three myeloid cell lines tested. Others, including *PLK3*, *PPP1CA*, and *PRKRA* were down-regulated in two of the three cell lines and *PPP2CA* was significantly down-regulated only in HL60 cells. Interestingly, the *p53* mRNA level was not altered by over-expression of miR-125b in the human NB4 cell line and the other human line tested, HL60, does not express *p53* (*data not shown*). Thus miR-125b expression inhibits apoptosis in different myeloid cell lines by down-modulating levels of different pro-apoptotic genes involved in the *p53* pathway rather than by down-regulation of a single gene.

The functions of miR-125b and its targets seem to be cell type-specific as miR-125b can be a tumor suppressor in some cancers, such as breast, liver or bladder cancers, but it acts as an oncogene in hematologic malignancies as it has been involved in myeloid and lymphoid leukemias.^{3,4,6-8,11,15,45}

In summary, we report that miR-125b is an oncomiR able to transform several human and murine myeloid cell lines. miR-125b blocks myeloid differentiation in part by targeting *CBFB*, blocks apoptosis through down-regulation of multiple genes involved in the *p53* pathway, and confers a proliferative advantage to human and mouse myeloid cell lines in part by targeting *ABTB1*. As miR-125b is deregulated in different hematologic malignancies, it could be a therapeutic target of choice in the treatment of certain leukemias.

Authorship and Disclosures

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