Attenuation of microRNA-126 Expression That Drives CD34⁺⁻38⁻ Stem/Progenitor Cells in Acute Myeloid Leukemia Leads to Tumor Eradication


Abstract

Despite high remission rates after therapy, 60% to 70% of patients with acute myeloid leukemia (AML) do not survive 5 years after their initial diagnosis. The main cause of treatment failures may be insufficient eradication of a subpopulation of leukemic stem-like cells (LSC), which are thought to be responsible for relapse by giving rise to more differentiated leukemic progenitors (LP). To address the need for therapeutic targets in LSCs, we compared microRNA (miRNA) expression patterns in highly enriched healthy CD34⁺⁻38⁻ hematopoietic stem cells (HSC), CD34⁺⁻38⁻ LSCs, and CD34⁺⁻38⁻ LPs, all derived from the same patients’ bone marrow (BM) specimens. In this manner, we identified multiple differentially expressed miRNAs, in particular miR-126, which was highly expressed in HSCs and increased in LSCs compared with LPs, consistent with a stem-like cell function. High miR-126 expression in AML was associated with poor survival, higher chance of relapse, and expression of genes present in LSC/HSC signatures. Notably, attenuating miR-126 expression in AML cells reduced in vitro cell growth by inducing apoptosis, but did not affect the survival of normal BM in which it instead enhanced expansion of HSCs. Furthermore, targeting miR-126 in LSCs and LPs reduced their clonogenic capacity and eliminated leukemic cells, again in the absence of similar inhibitory effects on normal BM cells. Our results define miR-126 as a therapeutic focus to specifically eradicate LSCs and improve AML outcome. Cancer Res; 74(7); 2094–105. ©2014 AACR.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous disorder that includes many entities with diverse genetic abnormalities and clinical features (1). Only a minority of cells within AML is responsible for sustaining and maintaining the leukemia (2). These leukemia-maintaining cells have many features in common with somatic normal stem cells and can self-renew and differentiate, which have given them the name "leukemic stem cells" (LSC). Although complete remissions are achieved, relapses occur often, which are thought to be due to survival of chemotherapy-resistant LSCs (3–5). Indeed, LSC frequency at diagnosis as well as after treatment and LSC and hematopoietic stem cell (HSC) gene expression signatures have been linked to AML outcome (3, 6, 7). Hypothetically, eradication of persistent LSCs will improve long-term AML outcome.

The intrinsic therapy resistance of LSCs together with their potential to (re-)initiate leukemia suggests that differences in gene expression, including microRNAs (miRNA), between LSCs and the bulk of the leukemia may include targets for anti-LSC therapy. Apart from LSCs, normal HSCs reside in the AML bone marrow (BM), necessitating development of anti-LSC therapy sparing HSCs. HSCs and LSCs share many features and the extent to which they differ will be instrumental for the development of LSC-targeted therapies without considerable toxicity. Searching for differences between LSCs and HSCs will be most relevant in cell fractions obtained from the same AML patients BM, taking into account the possible effects of the leukemic microenvironment on both stem cells (8).

Initially, LSCs capable of initiating human AML in immunodeficient nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice have been identified as having the CD34⁺⁻38⁻ phenotype, similar to HSCs (2). Later, leukemia initiating and maintaining capacity has been described in other immunophenotypically defined AML subpopulations (9, 10); however, immature CD34⁺⁻38⁻ cells still remain the best characterized and most potent population initiating leukemia in various xenograft mouse models and retransplantation experiments (2, 5, 7, 9, 10). To identify and purify LSCs and discriminate them from HSCs, we and others described leukemia-associated immunophenotypic markers (11–16). These markers include CLL1, CD123, CD47, CD96, Tim3, and lineage markers such as CD56 and CD7 (11–16). Moreover, we and...
others identified that in general leukemic CD34+/CD38− cells have lower aldehyde dehydrogenase (ALDH) activity than HSCs coexisting in the AML BM (17, 18). Importantly, ALDH activity can reliably distinguish leukemic CD34+/CD38− cells, capable of leukemic engraftment, from CD34+/CD38− HSCs, capable of multilineage engraftment (17, 18).

miRNAs are small, noncoding RNAs that control gene expression by repressing translation or by promoting degradation of target mRNAs (19). Virtually, all cancers are characterized by abnormal miRNA expression patterns, which in several cancers, including AML, strongly correlate with tumor classification, cytogenetic status, molecular abnormalities, and prognosis (20–22). Moreover, deregulated expression of miRNAs is associated with uncontrolled self-renewal and/or therapy resistance in hematologic malignancies (23–28). Because miRNAs target multiple genes, manipulation of their expression could potentially affect multiple pathways at once. In view of AML as a heterogeneous disease, and not successfully treated by targeting a single gene, this broad effect may hold the key to therapeutic success in AML. The potential of miRNAs to serve as LSC therapeutic targets has also been suggested by their ability to convert normal myeloid progenitors/stem cells into AML LSCs. For example, enhanced expression of miR-29a in normal hematopoietic cells resulted in a myeloproliferative disorder that progressed to AML (24). Knockdown of miR-126 results in reduced survival of AML cells, indicating the potential for the treatment of leukemia (27).

There are many studies determining the miRNA profiles of the bulk of primary AML cells (20–22, 29, 30), but identification of miRNA expression in LSCs, leukemic progenitors (LP), and HSCs obtained from the same AML BM has never been conducted. Here, we report for the first time the comparison between the expression of miRNAs in CD34+/CD38− LSCs and CD34+/CD38− LPs and between LSCs and HSCs all from the same AML BM. In this way, we identified multiple LSC- and HSC-specific miRNAs. One of the miRNAs with enhanced expression in LSCs that was compared with LPs is miR-126. Knockdown of miR-126 results in reduced survival of AML leukemic (stem) cells; however, it does not affect survival of normal hematopoietic (stem) cells, indicating the potential of targeting miR-126 for specific LSC therapy.

**Materials and Methods**

**Patient samples and AML cell lines**

Patient material was derived from patients with AML who were treated at the VU University Medical Center (VUMC), Amsterdam, the Netherlands, or in a hospital participating in the HOVON 42 or HOVON 102 AML trials (http://www.hovon.nl). Normal BM was obtained from cardiology patients undergoing cardiothoracic surgery. Informed consent was obtained for every used BM sample and the procedure was approved by the ethical committee of the VUMC. THP-1 and MV4-11 were purchased from the American Type Culture Collection. MM6 was obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ). All cell lines were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal calf serum, 1% l-glutamate (Invitrogen/Life Technologies), and 1% penicillin/streptomycin.

**Molecular diagnostics and cytogenetic analysis**

Mononuclear cells were isolated using Ficoll-Paque Plus (Amersham Biosciences) and DNA and/or RNA was studied for the presence of t(9;22), t(8;21), t(15;17) and mixed-lineage leukemia (MLL) translocations, CEBPα, FLT3-ITD, and NPM1 mutations, and the overexpression of EVI1 following standard procedures (www.mdhem.nl). Cytogenetics was performed according to standard techniques.

**ALDH activity and cell sorting**

ALDH activity was assayed using Aldefluor assay (Stem Cell Technologies). Cells were labeled with fluorochrome-conjugated antibodies as was previously described (18). Annexin V and/or 7-aminocoumarin D (7-AAD) were used as viability markers. Antibodies were purchased from BD Biosciences, Zebra Biosciences, Dako, or Sanquin. Analysis and purification by flow cytometry were done using a FACSAria (BD Biosciences). HSCs were defined as SSCintCD45dimCD34+ALDHdimmarker−. LSCs as SSCintCD45dimCD34+ALDHdimmarker+ and LPs as SSCintCD45dimCD34+ALDHdim/lowmarker+. LSCs carried an ALDHdim/low/CD38−/CD34+ phenotype.

**RNA isolation and miRNA microarray hybridization**

Total RNA was isolated with the NucleoSpin miRNA Kit (Macherey-Nagel) according to manufacturer’s protocol and concentrated using a vacuum concentrator (SPD111V; Thermo Savant). Human miRNA arrays (V3; Agilent Technologies), containing 15,000 probes representing 866 human and 89 human viral miRNAs (Sanger miRBase; release 12.0), were used. Deseasonalization, ligation, and hybridization were performed using the Agilent miRNA Complete Labeling and Hyb Kit (Agilent Technologies). Slides were scanned by a High-Resolution C Scanner (Agilent) and images were analyzed with Feature Extraction software, version 10.5.1.1. Normalization was done by the quantile method (31). The signal of all probes representing the same miRNA was averaged. MiRNAs were considered differentially expressed when the ratio was over or under the average of all ratios plus or minus the standard deviation.

**Quantitative real-time PCR analysis**

All reverse transcription (RT) and PCR reactions were performed according to manufacturer’s protocol (Applied Biosystems). RNU48 was used as a control gene. Low cell amount quantitative RT-PCR (qRT-PCR) was performed on 100 cells. Cells were snap-frozen and cDNA was generated by the MiRNA RT Kit (Applied Biosystems). Experiments were performed in duplicate and Ct values were averaged. For the PCR on 100 cells, expression was calculated using the 2−ΔCt method without normalization with a small RNA control. Statistical significance was determined using the two-sided paired Student t test.

**Survival analysis and gene expression analysis**

miRNA and mRNA sequencing results together with clinical data from approximately 200 patients with AML (32) were downloaded from https://tcga-data.nci.nih.gov/docs/publications/amr_2012 and analyzed with BRB-ArrayTools (version 4.2.0). Genes in which less than 20% of samples had
less than 2.5-fold change from the median value were excluded. This resulted in 1,896 genes, which were used in a Spearman rank correlation analysis with miR-126 expression, using a significance threshold of univariate tests <0.001. Correlated genes were compared with previously published HSC and LSC gene signatures (7).

For survival analysis, overall survival (OS), event-free survival (EFS), and relapse-free survival (RFS) were correlated with miR-126 expression in non–core binding factor (CBF) leukemias in patients <60 and >60 years of age. The top third highest miR-126–expressing patients with AML in each group were compared with the rest of AML cases. All statistical analysis were performed using SPSS 21.0 package (IBM SPSS Statistics). All statistical differences were considered significant at a P value of ≤0.05.

Lentivirus production and transduction of AML and normal BM cells

For production of miR-126 knockdown (KD) lentivirus, the miRZip lentiviral-based miRNA inhibitor plasmid (mZIP126-3p) was purchased from System Biosciences. As a control, the pGreenPuro Scramble Hairpin plasmid (mZIP000) was used. Viral particles were produced as previously described (33) and lentiviruses were concentrated using polyethylene glycol (34). Cell lines were transduced in the presence of polybrene (8 μg/mL; Sigma) with a multiplicity of infection (MOI) of 25. CD34+/CD38− normal and AML BM cells were isolated with flow cytometry or immunomagnetic beads. Primary AML and normal BM cells were incubated for 2 days in CellGro Stem Cell Growth Medium (SCGM; CellGenix) supplemented with rhIL3, rhFLT3-L, rhSCF, and 1% penicillin/streptomycin and Fungizone 0.125 μg/mL (Life Technologies). For the colony-forming unit (CFU) assay, normal BM cells were incubated for 2 days in CellGro Stem Cell Growth Medium (SCGM; CellGenix) supplemented with rhIL3, rhFLT3-L, rhSCF (and rhTPO for BM) before transduction with MOI ranging from 25 to 100.

Long-term liquid culture and colony-forming unit assay

For the long-term liquid culture (LT-LIC) assay, AML cells were cultured in CellGro SCGM with rhIL3, rhFLT3-L, rhSCF and 1% penicillin/streptomycin and Fungizone 0.125 μg/mL (Life Technologies). For the colony-forming unit (CFU) assay, cells were cultured in MethoCult with or without erythropoietin (Stemcell Technologies) for 14 days at 37°C.

Xenograft mouse model

NOD/SCID/IL2r γ (null) mice (NSG) were purchased from The Jackson Laboratory. The described research was approved by the Animal Care Committee of the VUMC (DEC-Hema-10-01). Six- to 9-week-old mice were injected subcutaneously in both flanks with 0.5 × 10^6 THP1 cells transduced with miR-126 KD or control vector. After the tumor became palpable, the size was measured every other day. Tumor volume was calculated by length × width × depth. When tumors reached a volume of 1,000 mm³, mice were euthanized and tumors were removed and weighted.

Results

Detection and purification of normal HSCs, LSCs, and LPs from AML BM

The activity of ALDH can be used to subdivide the total CD34+/CD38− stem cell compartment into a leukemic and a normal fraction. We used this distinctive property to purify LSCs and HSCs from the BM of patients with AML (17, 18). The ALDH<sup>high</sup> population in these patients contains the normal HSCs, devoid of immunophenotypical and molecular aberrancies. The ALDH<sup>low/dim</sup> population contains the LSCs (17, 18).

Because not every patient with AML has detectable HSCs and/or LSCs, we first analyzed a series of AML BM samples (n = 50) for presence of both leukemic and normal CD34<sup>+</sup>CD38<sup>−</sup> cells by using the ALDH activity assay in combination with presence or absence of an immunophenotypical leukemia-associated marker expressed on the particular AML. From this analysis, we selected six AML samples (patient characteristics in Supplementary Table S1; Fig. 1). In these cases, ALDH activity segregates the CD34<sup>+</sup>CD38<sup>−</sup> cells in two compartments, ALDH<sup>high</sup> and ALDH<sup>low/dim</sup> (Fig. 1B). Absence of CLL-1 (AML 1, 2, and 4), or a lineage marker (AML 3, 5) on ALDH<sup>high</sup> CD34<sup>+</sup>CD38<sup>−</sup> cells suggests that these cells are normal (Fig 1C). The ALDH<sup>low/dim</sup> CD34<sup>+</sup>CD38<sup>−</sup> cells are leukemic because these cells express leukemia-associated markers (Fig. 1C). From these 6 AML cases, LPs were purified as CD34<sup>−</sup>CD38<sup>−</sup> compartment.

Identification of miRNAs differentially expressed between LSCs, LPs, and HSCs

Comparison of the miRNA expression profiles of LSCs with that of LPs from the 6 patients with AML resulted in identification of 12 differentially expressed miRNAs (Table 1, in at least 5 of 6 patients; Supplementary Table S2, in at least 4 out of 6 patients). miR-127-4a, miR-886-3p, miR-1305, miR-18a, miR-1260, miR-1914<sup>+</sup>, and miR-93 were decreased in LSCs as compared with more differentiated LP cells. miR-126, miR-22, miR-126<sup>+</sup>, miR-335, and miR-150 showed enhanced expression in LSCs (Table 1). Notably, miR-126, miR-126<sup>+</sup>, and miR-22 were increased and miR-1274a and miR-1914<sup>+</sup> were decreased in expression in LSCs in all six AML cases.

Three arrays (AML 1, 4, and 5) hybridized with HSC RNA did not pass quality control due to limited amount of RNA and were excluded from further analysis. Expression analysis in the other three AML cases resulted in identification of miRNAs differentially expressed between CD34<sup>+</sup>CD38<sup>−</sup> LSCs and HSCs, both residing within the AML BM (Table 1, in 3 out of 3 patients; Supplementary Table S3, in at least 2 out of 3 patients).

miR-551b, miR-10a, miR-151-5p, miR-29b, miR-125b, miR-23b, miR-196b, and let-7c were decreased in LSCs as compared with HSCs. miRNAs that showed higher expression in LSCs compared with HSCs were miR-181b, miR-221, miR-21, miR-22, and miR-130a (Table 1).

Confirmation of the miRNA expression in LSCs, LPs, and HSCs by qRT-PCR

To confirm our array results, we first performed qRT-PCR on the same RNA as we used for array hybridization for several of the miRNAs differentially expressed between LSCs and LPs (Supplementary Fig. S1). In this way, we confirmed the differential expression between LSCs and LPs of miR-22, miR-126, miR-150, miR-335, and miR-886-3p. To validate the expression profile of the identified miRNAs, we performed qRT-PCR analysis on LSCs, LPs, and HSCs purified from an independent panel of AML cases, including three AML cases already used for array analysis (AML 1/3/4). qRT-PCR analysis confirmed the
expression profile of miR-126 (15 out of 18) and miR-146 (10 out of 11; identified as increased in 4 of 6 patients; Supplementary Table S2; Fig. 2A and B). Moreover, LSCs have lower expression of miR-146a than HSCs, as was reported before (Supplementary Table S3; ref. 26).

Furthermore, we confirmed the expression profile of several of the miRNAs identified as being higher or lower expressed in LSCs compared with HSCs (Fig. 2C and D). miR-21 is higher expressed in the leukemic than the normal stem cells (Fig. 2C, n = 5), miR-10a (n = 6), miR-125b (n = 6), and miR-551b (n = 11) are lower expressed in LSCs (Fig. 2C). In most AML cases, miR-551b and miR-10a are not or very low expressed in leukemic cells. We found that miR-181a is higher expressed in LSCs versus HSCs in 2 of 3 patients (Supplementary Table S3) in our initial array analysis and in 5 of 5 patients in our qRT-PCR analysis (Fig. 2D). A family member of miR-181a, miR-181b is also enhanced in LSCs compared with HSCs (3 of 3 patients; Table 1). Altogether, qRT-PCR analysis of miR-21, miR-181a, miR-125b, miR-10a, miR-551b, miR-126, miR-335, miR-150, miR-886-3p, and miR-146a in LSCs, HSCs, and LPs concomitantly present in the AML BM confirmed our results obtained by array expression analysis.

miRNA-126 is enhanced in HSCs and LSCs and its expression is associated with stem cell genes

Because miR-126 is the miRNA with the largest differential expression between LSCs and LPs, we selected this miRNA for further study. miR-126 is increased in LSCs compared with LPs (Fig. 2A) and even higher expressed in normal HSCs (16 out of 17 cases, Fig. 2E), suggesting miR-126 to be a stem cell–associated miRNA. If so, expression of miR-126 in AML might be associated with expression of stem cell genes and/or poor survival. To investigate this, we analyzed the gene expression signature associated with miR-126 in a panel of 200 patients with AML (32). Because miR-126 is highly expressed in CBF AML cases (Supplementary Table S4; refs. 21, 22), a subgroup of AML patients with a good prognosis, and thereby possibly influencing the correlation analysis, we excluded patients harboring a t(8;21) or inv(16). The Spearman rank correlation analysis resulted in 854 genes significantly coexpressed with miR-126 (Supplementary Table S5). As could be expected, the EGFL7 gene, in which the intragenic miR-126 is located, is the most positively correlated gene. To investigate whether miR-126 is coexpressed with genes present in HSCs and LSCs, we investigated whether these 854 genes are present in previously published HSC (HSC-R) and LSC (LSC-R) gene expression profiles (7). From the 130 genes [false discovery rate (FDR) < 0.05] present in the HSC-R signature, 30 genes overlapped with our miR-126 coexpressed genes. Of these genes, 23 were highly correlated (correlation coefficient >0.45), including MLLT3, BAALC, INPP4B, PROM1, CD109, ABCB1, and ERG. The published LSC-R signature consists of 219 genes (FDR < 0.1), of which 27 genes showed overlap.
with the miR-126 correlated genes. Of these, 18 were positively and 9 genes were negatively correlated with miR-126 expression. Among these were SLC9A7, ABCG1, MEF2C, RBMPS, LYZ, CSTA, and HAL.

miRNA-126 expression is associated with an adverse prognosis in AML

To investigate whether miR-126 expression levels are associated with the prognosis of AML patients, we
correlated results from miRNA sequencing data with clinical outcome of 92 patients with AML (>60 years of age; ref. 32). Because CBF AML has high miR-126 expression (Supplementary Table S4) and a good prognostic risk profile, we excluded AML cases belonging to this group (n = 16/108). The top 33% of AML cases with the highest miR-126 expression (n = 32) were compared with the rest of the AML cohort (n = 60). Patients with high miR-126 expression showed poorer EFS (HR, 1.895; P = 0.013), RFS (HR, 2.434; P = 0.002), and a trend toward poorer OS (HR, 1.635; P = 0.083) compared with patients with low miR-126 (Fig. 3A–C). Inclusion of CBF leukemias in the survival analysis resulted in an improved outcome of the “miR-126 high” AML group. The adverse effect of miR-126 expression on OS and EFS was thereby abolished and the impact on RFS (HR, 1.696; P = 0.052) was weaker. Of note, miR-126 did not show added value in CBF leukemias only, nor did it correlate with poor outcome in elderly patients (>60 years; data not shown).

Knockdown of miRNA-126 induces growth inhibition of AML cells by inducing apoptosis

To examine the functional role of miR-126 in AML (stem) cells, we decreased miR-126 in THP1 cells by lentiviral transduction with a miR-126 KD construct (mZip126-3p) containing green fluorescent protein (GFP; Fig. 4A). After transduction, the percentage of GFP-positive cells containing miR-126 KD decreased over time, indicating a decrease in growth rate upon miR-126 KD (Fig. 4B). Transduction with control plasmid did not result in a growth disadvantage. In a short-term assay, miR-126 KD resulted as well in decreased cell numbers (Fig. 4C). Downregulation of miR-126 resulted also in inhibition of growth in two other AML cell lines, MV4-11 and MM6 (Fig. 4D).

Inhibition of cell growth by downregulation of miR-126 can be due to inhibition of proliferation and/or induction of apoptosis. To examine the effect of miR-126 KD on proliferation, we labeled THP1 cells with PKH26 and showed that miR-126 KD cells have slightly more PKH26 at day 10 than the control cells (Fig. 4E). The estimated cell doubling time for...
miR-126 KD and the control cells was respectively 1.9 days (95% confidence interval, CI, 1.8–2.1 days) and 2.3 days (95% CI, 2.1–2.5 days). THP1 cells with decreased miR-126 have no change in cell-cycle state (Fig. 4F).

To examine if miR-126 plays a role in the induction of apoptosis, we decreased its expression with the miR-126 KD lentivirus and enhanced its expression with a miR-126 overexpression lentivirus in THP1 cells. Cells with decreased miR-126 levels showed twice as much apoptotic cells than control cells (Fig. 4G), whereas ectopic expression had no effect (Fig. 4G). Downregulation of miR-126 also induced cell death in MV4-11 and MM6 (Fig. 4H).

**miRNA-126 downregulation results in decreased leukemic growth in a xenotransplant mouse model**

To show the therapeutic potential of targeting miR-126 in vivo, we tested leukemic cells with decreased miR-126 expression for growth in a subcutaneous AML xenograft model. NSG mice were subcutaneously injected with THP1 cells with almost 10-fold decrease in miR-126 expression and THP1 control cells (Fig. 5A). Tumors of mice injected with THP1 miR-126 KD cells appeared later and reached the 1,000 mm³ later than tumors of control cells, resulting in prolonged survival (median survival, 18 vs. 21 days; P < 0.001; Fig. 5B). Correction of the tumor weight by the time between injection of the AML cells and removal of the tumor resulted in an estimated doubling time for each individual tumor (Fig. 5C). Mice injected with control cells had significantly (P = 0.006) faster growing tumors than those injected with miR-126 KD cells having doubling times of 2.00 days (±0.057) and 2.23 days, respectively (±0.052), P = 0.0052.

Knockdown of miRNA-126 decreases survival of leukemic stem and progenitor cells but spares normal HSCs

To investigate whether targeting of miR-126 could be a potential future AML LSC therapy, we purified CD34⁺ CD38⁻ LSCs and CD34⁺ CD38⁺ LPs from 2 patients with AML and transduced these cells with miR-126 KD and control lentivirus. CFU assays showed a decreased number of colonies after knockdown of miR-126 (1.7–5.3-fold decrease compared with control cells) in both patients with AML (Fig. 6A). Moreover, CD34⁺ CD38⁻ LPs also had reduced clonogenic capacity after miR-126 KD (3.0–4.2-fold decrease).

To determine whether the decrease in colony-forming capacity after miR-126 targeting is due to decreased survival and/or decreased clonogenic capacity, we performed CFU assays with GFP-positive or puromycin-selected AML CD34⁺ cells transduced with miR-126 KD or control virus. In GFP-positive and puromycin-selected cells, downregulation of miR-126 gave a reduced number of colonies, indicating the potential of miR-126 targeting to decrease clonogenic capacity (Fig. 6B).

Long-term culturing of primary AML cells can detect stem cells in vitro based on their ability to maintain progenitor cells with clonogenic potential over a period of 5 to 7 weeks. Progenitors cannot survive culturing for a long period and therefore long-term culturing will detect progenitors derived from stem cells present at the start of the experiment. Transduced CD34⁺ AML cells after 7 weeks of culturing showed no viable GFP-positive cells, whereas in the control samples, a viable GFP-positive cell compartment (6.1%–7.1%) was observed (Fig. 6C), indicating elimination of AML LSCs and progenitors upon miR-126 knockdown.

Because miR-126 is highly expressed in HSCs (Fig. 2E), knockdown of miR-126 in normal BM might be relatively harmful. Downregulation of miR-126 in CD34⁺ cells of normal BM and AML patients reduced the number of cells in all four cases (Fig. 6D); however, the decrease was more in AML than in normal BM (6.2- vs. 2.3-fold reduction). The observed reduction in cell number can be due to inhibition of proliferation or to induction of apoptosis. Importantly, only AML cells have induction of apoptosis after miR-126 knockdown (Fig. 6E).

Besides the induction of apoptosis, miR-126 could play a role in differentiation of LSCs and HSCs. To that end, we investigated whether the percentage of living CD34⁺ CD38⁻ stem cells, CD34⁻ CD38⁻ progenitors, and CD34⁻ CD38⁻ cells after miR-126 KD was changed in AML (n = 6) and normal BM (n = 10). Correction for sample variation was done by comparing GFP⁺ with GFP⁻ cells and ratios were calculated for miR-126 KD versus control cells, using the formula

\[
\frac{\%\text{GFPpos miR126KD}}{\%\text{GFPpos control}} / \frac{\%\text{GFPneg miR126KD}}{\%\text{GFPneg control}}
\]

Knockdown of miR-126 in normal BM resulted in increase of CD34⁺ CD38⁻ HSCs and CD34⁻ CD38⁻ progenitors (Fig. 6F). In contrast, miR-126 KD significantly reduced the frequency of CD34⁻ CD38⁻ LSCs and increased the CD34⁺ CD38⁻ progenitors in AML (Fig. 6F). In two AML cases, no CD34⁺ CD38⁻ cells could be detected after 5-day culture. To determine whether knockdown of miR-126 in normal BM is harmful to the
clonogenic and differentiation potential of HSCs and progenitors, CD34<sup>+</sup> BM cells transduced with miR-126 KD were tested for colony-forming capacity and differentiation potential. GFP<sup>+</sup> miR-126 KD cells showed similar colony-forming capacity as control cells with a similar distribution of all colony types (Fig. 6F). Colonies from miR-126 KD cells were smaller in size, probably reflecting their decreased proliferation.

Figure 4. Knockdown of microRNA-126 results in induction of apoptosis. A, qRT-PCR analysis of miR-126. B, coculture of THP1-GFP or miR-126 KD-GFP cells with wild-type THP1 cells. C, THP1 cells transduced with miR-126 KD and control plasmid were cultured and counted. D, miR-126 KD and control-transduced THP1, MM6, and MV4-11 were cultured for 5 days and cells were counted. E, THP1 cells with miR-126 KD or control vector labeled with PKH26 were measured for signal intensity by flow cytometry. F, cell-cycle analysis of THP1 cells transduced with miR-126 KD or control plasmid. G, THP1 AML cells with decreased and enhanced miR-126 expression were analyzed for apoptosis by Annexin V/7-AAD staining and flow cytometry. Percentage of positive cells in control THP1 was set to 1. H, viability analysis using LIVE/DEAD stain.
Discussion

Due to the difficulty in isolating sufficient numbers of pure stem cells, no study exists wherein differences in miRNA expression of LSCs, LPs, and HSCs of the same patient with AML are determined. Our study represents the first effort to simultaneously compare miRNA expression profiles of all these fractions from the same AML BM, which takes into account the influence of the AML microenvironment on the expression profiles.

In the six AML cases we used for miRNA profiling, the median HSC fraction was 0.115% (range, 0.02–1.26) of the total CD34+ compartment, which is in agreement with previously reported frequencies (0.12% in ref. 17). On the basis of the ratios between LSCs and LPs and between LSCs and HSCs, miRNA expression profiles of LSCs were, in general, more comparable with that of LPs than with that of HSCs. This is in accordance with gene expression profiles of LSCs, LPs, and HSCs (35).

Our miRNA profiling identified multiple miRNAs differentially expressed between LSCs and LPs. These miRNAs could play a role in establishing and maintaining the LSC state and may functionally influence stem cell properties such as quiescence, niche dependence, therapy resistance, and self-renewal. One of these miRNAs, miR-93, was lower expressed in LSCs than LPs. Interestingly, like in LSCs, miR-93 is also decreased in colon cancer and breast cancer stem cells (36, 37). In breast cancer, enforced expression of miR-93 targeted several stem cell regulatory genes resulting in depletion of cancer stem cells and inhibition of tumor development (36). The lower expression of miR-93 in LSCs might therefore also be partly responsible for LSCs survival. Interestingly, expression of miR-150 is lower in AML than in normal BM (38); however, LSCs show enhanced expression compared with the bulk of the AML.

Comparison of miRNA profiles of LSCs with that of HSCs resulted in identification of various miRNAs previously shown to play a role in normal hematopoietic stem/progenitor function and/or development of AML. miR-181a/b, miR-221, miR-22, and miR-107a are enhanced in AML compared with normal CD34+ cells (20–22), indicating their possible oncogenic function. Indeed, several of these miRNAs can function as oncogenes in leukemia; e.g., miR-21 targets PTEN that, upon deletion, can lead to myeloproliferative disease and leukemia in mice (39). Moreover, miR-21 knockout in myeloid cells resulted in an increased sensitivity to various chemotherapeutic agents (40). miR-221 also targets PTEN, as well as important genes like PUMA, FOXO3, and Bim (41). miR-181a/b has been reported to target HOXA genes, which in HSCs play an important role in stem cell maintenance (42). We found miR-125b, miR-10a, miR-196b, miR-551b, and miR-29b highly expressed in HSCs and to be decreased in AML, suggesting a potential role for these miRNAs in maintaining hematopoietic stem cell features such as self-renewal and/or therapy resistance. In fact, miR-196b, miR-29b, and miR-125b have been shown to be involved in the formation of leukemia (27, 43, 44).

Interestingly, miR-126 is highly expressed in HSCs and shows enhanced expression in LSCs compared with LPs, indicating a possible role for miR-126 in regulating hematopoietic as well as leukemic stem cell properties. Indeed, we show that patients with AML with high miR-126 levels coexpress genes that are also present in published HSC and LSC signatures (7). Moreover, these patients have a worse prognosis compared with patients with low miR-126 expression. Importantly, we show that knockdown of miR-126 in AML results in the induction of apoptosis and reduction of tumor growth in an AML xenograft mouse model.

Importantly, the targeting of miR-126 in AML decreased the CD34+CD38− compartment and reduced the clonogenic capacity of LSCs and LPs. In contrast to AML, and like the results obtained by Lechman and colleagues (45), we show that knockdown of miR-126 in normal CD34+ cells leads to CD34+CD38− expansion. We hypothesize that knockdown of miR-126 in LSCs and LPs within AML might result in a drop in miR-126 expression, leading to induction of apoptosis and/or differentiation. This will result in reduced colony-forming capacity. Because miR-126 expression levels are far higher in HSCs than in LSCs, knockdown of miR-126 in HSCs might result in a decrease in miR-126 levels, which is not sufficient for induction of apoptosis and HSCs survive. Alternatively, both LSCs and LPs might be dependent on miR-126 expression for survival, whereas normal HSCs may
The fact that miR-126 KD has apoptotic and anti-clonogenic effects in AML and gives an increase in HSC levels might even result in enhanced hematologic recovery, due to the expansion of long-term repopulating HSCs, after miR-126 knockdown in AML (45).

In conclusion, we identified miRNAs differentially expressed between CD34+CD38− primary AML cells and CD34+CD38− LPs as well as between LSCs and residual CD34+CD38− HSCs within the AML BM. We show that miR-126 expression is associated with stem cell–related genes and poor survival in AML, and that downregulation of miR-126 leads to induction of apoptosis and decreased clonogenic capacity of AML LSCs and LPs while sparing normal CD34+CD38− HSCs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: D.C. de Leeuw, G.J. Ossenkoppele, L. Smit
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.C. de Leeuw, A.P. Rutten, W. Pouwels, G.J. Schuurhuis, L. Smit
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.C. de Leeuw, M.C. Olthof, L. Smit
Writing, review, and/or revision of the manuscript: D.C. de Leeuw, G.J. Schuurhuis, G.J. Ossenkoppele, L. Smit
Study supervision: G. Jan Schuurhuis, G.J. Ossenkoppele, L. Smit

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 25, 2013; revised December 11, 2013; accepted December 28, 2013; published OnlineFirst January 29, 2014.
References


Attenuation of microRNA-126 Expression That Drives CD34+38− Stem/Progenitor Cells in Acute Myeloid Leukemia Leads to Tumor Eradication

David C. de Leeuw, Fedor Denkers, Marjolein C. Othof, et al.