Attenuation of microRNA-126 expression that drives CD34+38- stem/progenitor cells in acute myeloid leukemia leads to tumor eradication.

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Abstract

Despite high remission rates after therapy, 60-70% of acute myeloid leukemia (AML) patients do not survive five 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 LSC, we compared microRNA expression patterns in highly enriched healthy CD34+CD38- hematopoietic stem cells (HSC), CD34+CD38- LSC and CD34+CD38+ LP, all derived from the same patients' bone marrow specimens (BM). In this manner, we identified multiple differentially expressed microRNAs, in particular miR-126 which was highly expressed in HSC and increased in LSC compared to LP, 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 where it instead enhanced expansion of HSC. Furthermore, targeting miR-126 in LSC and LP 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 LSC and improve AML outcome.
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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 LSC (3,4,5). 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 LSC will improve long-term AML outcome.

The intrinsic therapy resistance of LSC together with their potential to (re-)initiate leukemia suggests that differences in gene expression, including microRNAs (miRNAs), between LSC and the bulk of the leukemia may include targets for anti-LSC therapy. Apart from LSC, normal HSC reside in the AML BM necessitating development of anti-LSC therapy sparing HSC. HSC and LSC 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 LSC and HSC 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, LSC capable of initiating human AML in immunodeficient NOD/SCID mice have been identified as having the CD34+CD38- phenotype, similar to HSC (2). Later, leukemia initiating and maintaining capacity has been described in other immunophenotypically defined AML subpopulations (9,10), however immature CD34+CD38- cells still remain the best characterized and most potent population initiating leukemia in various xenograft mouse models and re-transplantation experiments (2,5,7,9,10). To identify and purify LSC and discriminate them from HSC 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
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identified that in general leukemic CD34+CD38- cells have lower aldehyde dehydrogenase (ALDH) activity than HSC co-existing in the AML BM (17,18). Importantly, ALDH activity can reliably distinguish leukemic CD34+CD38- cells, capable of leukemic engraftment, from CD34+CD38- HSC, 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 hematological malignancies (23-28). Since 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 LSC. For example, enhanced expression of miR-29a in normal hematopoietic cells resulted in a myeloproliferative disorder that progressed to AML (24) and enforced expression of solely miR-125b caused 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 LSC, LP and HSC 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- LSC and CD34+CD38+ LP and between LSC and HSC 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 LSC compared to LP is miR-126. Knockdown of miR-126 results in reduced survival of AML leukemic (stem) cells however does not affect survival of normal hematopoietic (stem) cells, indicating the potential of targeting targeting miR-126 for specific LSC therapy.

**Materials and Methods**


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Patient samples and AML cell lines

Patient material was derived from AML patients 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 trails (http://www.hovon.nl). Normal BM was obtained from cardiology patients undergoing cardiothoracal 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 (ATCC). 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 (FCS), 1% L-glutamate (Invitrogen Life Technologies) and 1% Penicillin/Streptomycin (P/S).

Molecular diagnostics and cytogenetic analysis

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

Aldehyde dehydrogenase activity and cell sorting

ALDH activity was assayed using Aldefluor™ assay (Stem Cell Technologies, Grenoble, France). Cells were labeled with fluorochrome-conjugated antibodies as was previously described (18). Annexin-V and/or 7-Amino-actinomycin D (7-AAD) were used as viability markers. Antibodies were purchased from: BD Biosciences, Zebra biosciences (Enschede, The Netherlands), Dako (Glostrup, Denmark) or Sanquin (Amsterdam, The Netherlands). Analysis and purification by flow cytometry was done using a FACS ARIA (BD Biosciences, Franklin Lakes, NJ, USA). HSC were defined as SSC<sub>low</sub>CD45<sub>dim</sub>CD34<sup>-</sup>CD38<sup>-</sup>
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ALDH\textsuperscript{bright} marker\textsuperscript{neg}, LSC as SSC\textsuperscript{low} CD45\textsuperscript{dim} CD34\textsuperscript{+} CD38\textsuperscript{-} ALDH\textsuperscript{dim} marker\textsuperscript{pos} and LP as SSC\textsuperscript{low} CD45\textsuperscript{dim} CD34\textsuperscript{+} CD38\textsuperscript{+} ALDH\textsuperscript{dim/low} marker\textsuperscript{pos}.

RNA isolation and miRNA microarray hybridization

Total RNA was isolated with the NucleoSpin miRNA kit (Macherey-Nagel, Düren, Germany) according to manufacturer's protocol and concentrated using a vacuum concentrator (SPD111V, Thermo Savant). Human miRNA arrays (V3) (Agilent Technologies; Palo Alto, CA), containing 15,000 probes representing 866 human and 89 human viral miRNAs (Sanger miRBase release 12.0), were used. Dephosphorylation, ligation and hybridization were performed using Agilent miRNA Complete Labeling and Hyb Kit (Agilent Technologies; Palo Alto, CA). Slides were scanned by a High-Resolution C Scanner (Agilent) and images were analyzed with Feature Extraction TM 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, Nieuwerkerk a/d IJssel, The Netherlands). RNU48 was used as a control gene. Low cell amount 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 $2^{-\Delta CT}$ method without normalization with a small RNA control. Statistical significance was determined using two-sided paired Student's t test.

Survival analysis and gene expression analysis
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MiRNA and mRNA sequencing results together with clinical data from ~200 AML patients (34) were downloaded from https://tcga-data.nci.nih.gov/docs/publications/aml_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 1896 genes which were used in a Spearman's 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) was correlated with miR-126 expression in non-CBF leukemia's in patients ≤60 years of age and >60 years of age. The top third highest miR-126 expressing AML patients in each group were compared with the rest of AML cases. All statistical analysis were performed using SPSS 21.0 package (IBM SPSS Statistics for Windows, Version 20.0, Armonk, NY, USA), with significance set at p≤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 (SBI)(San Francisco, CA). As a control the pGreenPuro scrambled hairpin plasmid (mZIP000) was used. Viral particles were produced as previously described (35) and lentiviruses were concentrated using PEG (36). Cell lines were transduced in the presence of polybrene (8ug/ml)(Sigma) with a multiplicity of infection (MOI) of 25. CD34+ 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™ SCGM (CellGenix) supplemented with rhIL3, rhFLT3-L, rhSCF (and rhTPO for BM) before transduction with MOI ranging from 25-100.

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% P/S and Fungizone 0.125ug/ml (Life
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Technologies). For the colony forming unit (CFU) assay, cells were cultured in MethoCult™ with (normal BM) or without EPO (AML)(Stemcell Technologies) for 14 days at 37°C.

Xenograft mouse model

NOD/SCID/IL2r gamma (null) mice (NSG) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The described research was approved by the Animal Care Committee of VUMC (DEC-Hema10-01). Six- to nine-week old mice were injected subcutaneously in both flanks with 0.5x10⁶ 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 x width x depth. When tumors reached a volume of 1000 mm³ mice were euthanized and tumors were removed and weighted.

Results

Detection and purification of normal HSC, LSC and LP 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 LSC and HSC from the BM of AML patients (17,18). The ALDH<sup>high</sup> population in these patients contains the normal HSC, devoid of immunophenotypical and molecular aberrancies. The ALDH<sup>low/dim</sup> population contains the LSC (17,18). Since not every AML patient has detectable HSC and/or LSC we first analyzed a series of AML BM samples (n>50) for presence of both leukemic and normal CD34+CD38- 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 supplemental Table 1)(Figure 1). In these cases, ALDH activity segregates the CD34+CD38- cells in two compartments, ALDH<sup>high</sup> and ALDH<sup>low/dim</sup> (Figure 1B). Absence of CLL-1 (AML 1, 2 and 4), or a lineage marker (AML 3, 5) on ALDH<sup>high</sup> CD34+CD38- cells suggests that these cells are normal (Figure 1C). The ALDH<sup>low/dim</sup> CD34+CD38- cells are
leukemic since these cells express leukemia-associated markers (Figure 1C). From these 6 AML cases LP were purified as CD34+CD38+ compartment.

**Identification of miRNAs differentially expressed between LSC, LP and HSC.**

Comparison of the miRNA expression profiles of LSC with that of LP from the six AML patients resulted in identification of 12 differentially expressed miRNAs (Table 1A [in at least 5 out of 6 patients], Supplemental Table 2 [in at least 4 out of 6 patients]). MiR-1274a, miR-886-3p, miR-1305, miR-18a, miR-1260, miR-1914* and miR-93 were decreased in LSC as compared to more differentiated LP cells. MiR-126, miR-22 miR-126*, miR-335 and miR-150 showed enhanced expression in LSC (Table 1A). Notably, miR-126, miR-126* and miR-22 were increased and miR-1274a and miR-1914* were decreased in expression in LSC in all six AML cases.

Three arrays (AML1, 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+CD38- LSC and HSC both residing within the AML BM (Table 1B [in 3 out of 3 patients], Supplemental Table 3 [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 LSC as compared to HSC. MiRNAs that showed higher expression in LSC compared to HSC were; miR-181b, miR-221, miR-21, miR-22 and miR-130a (Table 1B).

**Confirmation of the miRNA expression in LSC, LP and HSC 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 LSC and LP (Supplemental Figure 1). In this way we confirmed the differential expression between LSC and LP 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 in LSC, LP and HSC.
purified from an independent panel of AML cases, including three AML cases already used for array analysis (AML1/3/4). 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 4/6 patients; Supplemental Table 2)(Figure 2A, 2B). Moreover, LSC have lower expression of miR-146a than HSC, as was reported before (Supplemental Table 3, 26).

Furthermore, we confirmed the expression profile of several of the miRNAs identified as being higher or lower expressed in LSC compared to HSC (Figure 2C and 2D). MiR-21 is higher expressed in the leukemic than the normal stem cells (Figure 2C, n=5). MiR-10a (n=6), miR-125b (n=6), and miR-551b (n=11) are lower expressed in LSC (Figure 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 LSC versus HSC in 2/3 patients [supplemental Table 3] in our initial array analysis and in 5/5 patients in our qRT-PCR analysis (Figure 2D). A family member of miR-181a, miR-181b is also enhanced in LSC compared to HSC (3/3 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 LSC, HSC and LP concomitantly present in the AML BM confirmed our results obtained by array expression analysis.

MiRNA-126 is enhanced in HSC and LSC and its expression is associated with stem cell genes.

Since miR-126 is the miRNA with the largest differential expression between LSC and LP we selected this miRNA for further study. MiR-126 is increased in LSC compared to LP (Figure 2A) and even higher expressed in normal HSC (16 out of 17 cases, Figure 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 analysed the gene expression signature associated with miR-126 in a panel of 200 AML patients (34). Since miR-126 is highly expressed in core binding factor (CBF) AML
MicroRNA 126 is increased in leukemic stem cells (21,22)(Supplemental Table 4), a subgroup of AML patients with a good prognosis thereby possibly influencing a correlation with expression of stem cell genes we excluded patients harbouring a t(8;21) or inv(16) (n=15). Spearman's rank correlation analysis resulted in 854 genes significantly co-expressed with miR-126 (Supplemental Table 5). 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 co-expressed with genes present in HSC and LSC 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 (FDR<0.05) present in the HSC-R signature 30 genes overlapped with our miR-126 co-expressed 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. 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 AML patients prognosis we correlated results from miRNA sequencing data with clinical outcome of 92 AML patients (≤60 years of age)(34). Since CBF AML has high miR-126 expression (Supplemental table 4) and a good prognostic risk profile we excluded AML cases belonging to this group (n=13/105). The top 33% of AML cases with the highest miR-126 expression (n=23) were compared with the rest of the AML cohort (n=69). Patients with high miR-126 expression showed poorer EFS (Hazard ratio (HR) 1.895, p=0.013), RFS (HR 2.434, p=0.003) and a trend towards poorer OS (HR 1.635, p=0.086) compared to patients with low miR-126 (Figure 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,
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*p=0.052) 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)(Figure 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 (Figure 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 (Figure 4C). Downregulation of miR-126 resulted also in inhibition of growth in two other AML cell lines, MV4-11 and MM6 (Figure 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 and showed that miR-126-KD cells have slightly more PKH26 at day 10 than the control cells (Figure 4E). The estimated cell doubling time for miR-126 KD and the control cells was respectively 1.9 days (95% 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 (Figure 4F).

To examine if miR-126 plays a role in apoptosis we decreased its expression with the miR-126-KD lentivirus and enhanced its expression with a miR-126 overexpression (OE) lentivirus in THP1 cells. Cells with decreased miR-126 levels showed twice as much apoptotic cells than control cells (Figure 4G) whereas ectopic expression had no effect (Figure 4G). Downregulation of miR-126 also induced cell death in MV4-11 and MM6 (Figure 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 THP-1 control cells (Figure 5A). Tumors of mice injected with THP1 miR-126KD cells appeared later and reached the 1000 mm$^3$ later than tumors of control cells, resulting in prolonged survival (median survival 18 vs. 21 days, p<0.001)(Figure 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 (Figure 5C). Mice injected with control cells had significantly (p=0.005) 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 HSC.

To investigate whether targeting of miR-126 could be a potential future AML LSC therapy we purified CD34+CD38- LSC and CD34+CD38+ LP from two AML patients 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 to control cells) in both AML patients (Figure 6A). Moreover, CD34+CD38+ LP 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
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a reduced number of colonies, indicating the potential of miR-126 targeting to decrease clonogenic capacity (Figure 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 5-7 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 showed after 7 weeks of culturing no viable GFP positive cells while in the control samples a viable GFP-positive cell compartment (6.1-7.1%). was observed (Figure 6C) indicating elimination of AML LSC and progenitors upon miR-126 knockdown.

Since miR-126 is highly expressed in HSC (Figure 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 (Figure 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 (Figure 6E).

Beside the induction of apoptosis, miR-126 could play a role in differentiation of LSC and HSC. To that end, we investigated whether the percentage of living CD34+CD38- stem cells, CD34+CD38+ progenitors and CD34- 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+ to GFP- cells and ratios were calculated for miR-126KD versus control cells, using the formula: Kp/Kc = %GFPpos miR126KD/%GFPneg miR126KD / %GFPpos control/%GFPneg control. Knockdown of miR-126 in normal BM resulted in increase of CD34+CD38- HSC and CD34+CD38+ progenitors (Figure 6F). In contrast, miR-126 KD significantly reduced the frequency of CD34+CD38- LSC and increased the CD34+CD38+ progenitors in AML (Figure 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 HSC and progenitors, CD34+ BM cells transduced with miR-126 KD knockdown were tested for colony forming capacity and differentiation potential. GFP+ miR-126 KD cells showed similar colony forming capacity and
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colonies with a similar distribution of all colony types (Figure 6F). Colonies from miR-126 KD cells were smaller in size, probably reflecting their decreased proliferation.

Discussion

Due to the difficulty in isolating sufficient numbers of pure stem cells no study exists wherein differences in miRNA expression of LSC, LP and HSC of the same AML patient is 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). Based on the ratios between LSC and LP and between LSC and HSC, miRNA expression profiles of LSC were, in general, more comparable to that of LP than to HSC. This is in accordance with gene expression profiles of LSC, LP and HSC (38).

Our miRNA profiling identified multiple miRNAs differentially expressed between LSC and LP. 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 LSC than LP. Interestingly, like in LSC, mir-93 is also decreased in colon cancer and breast cancer stem cells (39,40). 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 (38). The lower expression of miR-93 in LSC might therefore also be partly responsible for LSC survival (3,6). Interestingly, expression of miR-150 is lower in AML than in normal BM (41) however LSC show enhanced expression compared to the bulk of the AML.

Comparison of miRNA profiles of LSC with that of HSC resulted in identification of various miRNAs previously shown to play a role in normal hematopoietic stem/progenitor function
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and/or development of AML. MiR-181a/b, miR-221, miR-21, miR-22 and miR-130a are enhanced in AML compared to 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 (42). Moreover, miR-21 knockdown in myeloid cells resulted in an increased sensitivity to various chemotherapeutic agents (43). MiR-221 also targets PTEN, as well as important genes like PUMA, FOXO3 and Bim (44). MiR-181a/b has been reported to target HOXA genes which in HSC play an important role in stem cell maintenance (45). We found miR-125b, miR-10a, miR-196b, miR-551b and miR-29b highly expressed in HSC 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 (27,46,47).

Interestingly, miR-126 is highly expressed in HSC and shows enhanced expression in LSC compared to LP, indicating a possible role for miR-126 in regulating hematopoietic as well as leukemic stem cell properties. Indeed we show that AML patients with high miR-126 levels co-express genes that are also present in published HSC and LSC signatures (7). Moreover, these patients have a worse prognosis compared to patients with low miR-126 expression. Importantly, we show that knockdown of miR-126 in AML results in the induction of apoptosis and that it can reduce 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 LSC and LP. In contrast to AML, and like the results obtained by Lechman et al (48) we show that knockdown of miR-126 in normal CD34+ cells leads to CD34+CD38- expansion. We hypothesize that knockdown of miR-126 in LSC and LP 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. Since miR-126 expression levels are far higher in HSC than in LSC knockdown of miR-126 in HSC might result in a decrease in miR-126 levels which is not sufficient for induction of apoptosis and
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HSC survive. Alternatively, both LSC and LP might be dependent on miR-126 expression for survival while normal HSC may not. 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 hematological recovery, due to the expansion of long-term repopulating HSC, after miR-126 knockdown in AML (48).

In conclusion, we identified miRNAs differentially expressed between CD34+CD38- LSC and CD34+CD38+ LP as well as between LSC and residual CD34+CD38- HSC 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 LSC and LP while sparing normal CD34+CD38- HSC.

References


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Table 1

<table>
<thead>
<tr>
<th>Ratio LSC/Progenitor</th>
<th>AML1</th>
<th>AML2</th>
<th>AML3</th>
<th>AML4</th>
<th>AML5</th>
<th>AML6</th>
<th>Average</th>
<th>Number of patients</th>
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</thead>
<tbody>
<tr>
<td>hsa-miR-1274a</td>
<td>0.801</td>
<td>0.887</td>
<td>0.941</td>
<td>0.850</td>
<td>0.862</td>
<td>0.983</td>
<td>0.887</td>
<td>6/6</td>
</tr>
<tr>
<td>hsa-miR-886-3p</td>
<td>0.880</td>
<td>0.922</td>
<td>0.995</td>
<td>0.849</td>
<td>0.855</td>
<td>0.978</td>
<td>0.913</td>
<td>5/6</td>
</tr>
<tr>
<td>hsa-miR-1305</td>
<td>0.944</td>
<td>0.853</td>
<td>0.995</td>
<td>0.917</td>
<td>0.910</td>
<td>0.968</td>
<td>0.931</td>
<td>5/6</td>
</tr>
<tr>
<td>hsa-miR-18a</td>
<td>0.964</td>
<td>0.799</td>
<td>0.949</td>
<td>0.945</td>
<td>1.003</td>
<td>0.979</td>
<td>0.940</td>
<td>5/6</td>
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<tr>
<td>hsa-miR-1260</td>
<td>0.929</td>
<td>0.799</td>
<td>0.949</td>
<td>0.957</td>
<td>0.902</td>
<td>1.000</td>
<td>0.942</td>
<td>5/6</td>
</tr>
<tr>
<td>hsa-miR-126*</td>
<td>0.971</td>
<td>0.909</td>
<td>0.981</td>
<td>0.940</td>
<td>0.942</td>
<td>0.975</td>
<td>0.953</td>
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<tr>
<td>hsa-miR-93</td>
<td>0.978</td>
<td>0.861</td>
<td>0.996</td>
<td>0.971</td>
<td>0.944</td>
<td>1.000</td>
<td>0.954</td>
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<tr>
<td>hsa-miR-150</td>
<td>1.039</td>
<td>0.993</td>
<td>1.029</td>
<td>1.025</td>
<td>1.060</td>
<td>1.022</td>
<td>1.028</td>
<td>5/6</td>
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<tr>
<td>hsa-miR-335</td>
<td>1.070</td>
<td>0.954</td>
<td>1.063</td>
<td>1.032</td>
<td>1.052</td>
<td>1.052</td>
<td>1.037</td>
<td>5/6</td>
</tr>
<tr>
<td>hsa-miR-126*</td>
<td>1.032</td>
<td>1.079</td>
<td>1.028</td>
<td>1.083</td>
<td>1.048</td>
<td>1.083</td>
<td>1.059</td>
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<tr>
<td>hsa-miR-22</td>
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<td>1.154</td>
<td>1.037</td>
<td>1.084</td>
<td>1.062</td>
<td>1.024</td>
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<tr>
<td>hsa-miR-126</td>
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<td>1.386</td>
<td>1.048</td>
<td>1.076</td>
<td>1.048</td>
<td>1.058</td>
<td>1.115</td>
<td>6/6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ratio LSC/HSC</th>
<th>AML2</th>
<th>AML3</th>
<th>AML6</th>
<th>Average</th>
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<tbody>
<tr>
<td>hsa-miR-551b</td>
<td>0.681</td>
<td>0.681</td>
<td>0.717</td>
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<td>hsa-miR-10a</td>
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<td>hsa-miR-151-5p</td>
<td>0.805</td>
<td>0.799</td>
<td>0.820</td>
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<td>hsa-miR-29b</td>
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<td>0.869</td>
<td>0.888</td>
<td>0.841</td>
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<td>hsa-miR-125b</td>
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<td>0.909</td>
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<td>hsa-miR-23b</td>
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<td>0.859</td>
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<td>0.873</td>
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<td>hsa-miR-196b</td>
<td>0.916</td>
<td>0.868</td>
<td>0.846</td>
<td>0.877</td>
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<td>hsa-let-7c</td>
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<td>0.946</td>
<td>0.915</td>
<td>0.931</td>
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<td>hsa-miR-130a</td>
<td>1.069</td>
<td>1.124</td>
<td>1.121</td>
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<td>hsa-miR-22</td>
<td>1.262</td>
<td>1.121</td>
<td>1.095</td>
<td>1.157</td>
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<td>hsa-miR-21</td>
<td>1.237</td>
<td>1.081</td>
<td>1.242</td>
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<td>hsa-miR-221</td>
<td>1.073</td>
<td>1.191</td>
<td>1.456</td>
<td>1.24</td>
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<tr>
<td>hsa-miR-181b</td>
<td>1.217</td>
<td>1.123</td>
<td>1.488</td>
<td>1.276</td>
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</table>
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Legends

Figure 1: Purification of HSC, LSC and leukemic progenitors from AML samples. A) The stem cell compartment was defined as CD34+CD38- (black gate) and leukemic progenitors were defined as CD34+CD38+ (green gate). B) ALDH activity of CD34+CD38- cells shows two distinct populations; one with high ALDH activity (blue) and one with intermediate/low activity (red). C) CD34+CD38-ALDH<sup>high</sup> (HSC) and CD34+CD38-ALDH<sup>int/low</sup> (LSC) cells plotted against the LSC marker expressed in that particular AML case. In AML6, CLL-1 labeling was performed in a separate experiment.

Table 1: MicroRNAs differentially expressed between LSC and leukemic progenitors and HSC. A) MicroRNA expression ratios between CD34+CD38-ALDH<sub>low/dim</sub> (LSC) and CD34+CD38-ALDH<sub>high</sub> (HSC) populations in AML patients. The number of patients indicates the number with a significant differential expression for that microRNA. Italic printed ratios represent a non-significant difference. The average ratio is calculated for 6 AML patients. B) MicroRNA expression ratios between CD34+CD38-ALDH<sub>low/dim</sub> (LSC) and CD34+CD38-ALDH<sub>low/dim</sub> (LP) populations. The average ratio is calculated from 3 patients. In A and B, colors represent strength of the ratio in an individual patient. More intense red; higher expressed in LSC, more intense blue; higher expression in LP (A) or in HSC (B).

Figure 2: qRT-PCR analysis of miRNAs in LSC, HSC and LP. (A) MiR-126 expression analysis in LSC and LP of 18 AML cases. B) MiR-146a expression analysis in LSC and LP of 11 AML cases. C) Expression analysis of miR-21, miR-10a, miR-125b and miR-551b in LSC and HSC of AML patients D) Expression analysis of miR-181a in LSC and HSC of AML patients E) Expression analysis of miR-126 in LSC and HSC of 18 AML patients
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Figure 3: MiRNA-126 is associated with adverse prognosis in AML. Kaplan Meier survival curves of 92 non-CBF AML patients ≤60 years of age. A) Overall survival (OS) and B) event free survival (EFS). C) Relapse free survival (RFS), defined as, relapse or progression of disease after achieving CR (n=78).

Figure 4: Knockdown of miRNA-126 results in induction of apoptosis. A) QRT-PCR analysis of miR-126. B) Co-culture of THP-1-GFP or miR-126 KD-GFP cells with wild-type THP1 cells. C) THP-1 cells with miR-126-KD and control transduced THP1 cells 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 AnnexinV/7AAD staining and flow cytometry. Percentage of positive cells in control THP1 was set to 1. H) Viability analysis using LIVE/DEAD stain.

Figure 5: A) MiR-126 expression analysis by qRT-PCR  B) NSG mice subcutaneously injected with THP1 cells transduced with miR-126 KD (n=8) or control plasmid (n=7). Tumor growth was measured and mice were sacrificed when tumors reached a volume of 1000 mm3. C) Doubling time of the THP1 cells. Calculated based on tumor weight in combination with the day after AML cell injection at which mice were sacrificed.

Figure 6: Knockdown of miRNA-126 decreases survival of leukemic (stem and progenitor) cells but spares normal HSC. A) CFU assay of lentivirally transduced LSC and LP fractions from two AML patients. B) CFU assay of purified (GFP (left) or puromycin (right)) viable lentivirally transduced CD34+ primary AML cells C) 7 weeks culture of miR-126-KD transduced CD34+ AML cells (two cases) in liquid culture medium. D) Viable cell counts from two nBM and two AML samples that were lentivirally transduced with control vector or mi-126 KD 5 days after transduction. E) Levels of apoptosis measured by staining with
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AnnexinV 6 days after transduction. F) Normal BM (n=10) and AML (n=6) samples were lentivirally transduced with control vector or miR-126-KD and after 5 days percentage of GFP+ and GFP- cells in the CD34+CD38-, CD34+CD38+ and CD34- compartment was measured. Ratios between GFP+ and GFP- in control samples were set to 1. G) CFU assays of purified GFP+ CD34+ nBM cells transduced with control or miR-126-KD. GM, granulocytes and macrophages; G, granulocytes; M, macrophages; E, burst forming units (BFU) and CFU of erythrocytes.
Figure 3

A

Percent survival

miR-126 low (n=60)
miR-126 high (n=32)

p=0.083

OS (months)

B

Percent survival

miR-126 low (n=60)
miR-126 high (n=32)

p=0.01

EFS (months)

C

Percent survival

miR-126 low (n=53)
miR-126 high (n=25)

p=0.002

RFS (months)
Attenuation of microRNA-126 expression that drives CD34+38- stem/progenitor cells in acute myeloid leukemia leads to tumor eradication

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