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Targeting Leukemia Stem Cells *in vivo* with AntagomiR-126 Nanoparticles in Acute Myeloid Leukemia

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Abstract

Current treatments for acute myeloid leukemia (AML) are designed to target rapidly dividing blast populations with limited success in eradicating the functionally distinct leukemia stem cell (LSC) population, which is postulated to be responsible for disease resistance and relapse. We have previously reported high miR-126 expression levels to be associated with a LSC-gene expression profile. Therefore, we hypothesized that miR-126 contributes to “stemness” and is a viable target for eliminating the LSC in AML. Here we first validate the clinical relevance of miR-126 expression in AML by showing that higher expression of this microRNA (miR) is associated with

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worse outcome in a large cohort of older (≥60 years) cytogenetically normal AML patients treated with conventional chemotherapy. We then show that miR-126 overexpression characterizes AML LSC-enriched cell subpopulations and contributes to LSC long-term maintenance and self-renewal. Finally, we demonstrate the feasibility of therapeutic targeting of miR-126 in LSCs with novel targeting nanoparticles (NP) containing antagomiR-126 resulting in *in vivo* reduction of LSCs likely by depletion of the quiescent cell subpopulation. Our findings suggest that by targeting a single miR, i.e., miR-126, it is possible to interfere with LSC activity, thereby opening potentially novel therapeutic approaches to treat AML patients.

Keywords

miR-126; leukemia stem cell; antagomiR-126

Introduction

Acute myeloid leukemia (AML) is a clonal, neoplastic disease that is heterogeneous at the molecular, cytogenetic, cellular and clinical levels[1, 2]. While advances have been made towards understanding the biology of AML, effective and relatively non-toxic treatments are still lacking and the prognosis is poor. The occurrence of multiple mutations and dysregulated gene expression contributes to the biologic and clinical complexity of AML and impact on patients' treatment response and overall prognosis[3]. Furthermore, our view of a morphologically homogenous and functionally static blast population present in the bone marrow and blood of individual AML patients has now evolved into our current perspective of multiple dynamic and heterogeneous cell subpopulations including the relatively rare leukemia initiating cells [i.e., leukemia stem cells (LSCs)] [4, 5]. These cells have acquired abnormal self-renewal and partial maturation ability and are considered responsible for disease initiation and maintenance[6]. Thus, targeting LSCs may represent an essential step for complete eradication of the disease. However, LSCs are resistant to conventional chemotherapy regimens, and novel approaches are needed to eliminate these cells and improve clinical outcome[7, 8].

MicroRNAs (miRs) are small non-coding RNA molecules that regulate gene expression at the mRNA, DNA, and/or protein level[9]. miRs for the most part regulate mRNA expression by hybridizing to the 3′-UTR sequence of mRNAs, resulting in down-regulation of target genes[9, 10]. This mechanism has been shown to play an important regulatory role for both normal and malignant hematopoiesis[11, 12], and altered expression of miRs has been associated with clinical outcome in AML[3, 13-17].

In normal hematopoiesis, miR-126 was found to be expressed in hematopoietic stem cells (HSCs) and early hematopoietic progenitor cells (HPCs) and to regulate HSC growth and activation [18]. Furthermore, our group previously reported that high expression of miR-126 is associated with a LSC-enriched gene expression profile in cytogenetically normal (CN) AML[1]. Altogether these data support a role of miR-126 in myeloid leukemogenesis and suggest this miR as a potentially novel therapeutic target for AML LSCs.

We demonstrate that miR-126 knock-down decreased the number of LSCs by impairing stem cell self-renewal as determined by long-term colony initiating cell (LTC-IC) and colony forming cell (CFC) re-plating assays along with depletion of the quiescent cell sub-fraction. Efficient antagomiR-126 delivery using nanoparticles conjugated to antibodies binding antigens present on LSCs, resulted in *in vivo* miR-126 knock-down and depletions of LSCs thereby leading to longer survival of leukemic mice in secondary transplant experiments. Altogether, these data support miR-126 as a novel therapeutic target to impact LSC activity in AML.

Material and Methods

Primary cells, miR-126 expression and methylation quantification

See supplemental methods

RNA Extraction, RNA Expression Quantification

RNA, cDNA, and real time PCR was performed using previously published methods (see also supplemental methods)[20].

Transferrin or anti-CD45.2 antibody conjugated nanoparticle preparation

Previously we developed a transferrin targeted neutral nanoparticle delivery system[21]. Briefly, positively charged polyethylenimine and negatively charged antagomiRs, anti-miR hsa-miR-126-3p cat#AM17004 (Ambion, Austin, TX) or anti-miR-scramble (SCR) cat#AM17010, (Ambion Austin, TX) form a polyplex core. This core was then loaded to pre-made anionic liposomal nanoparticles to form lipopolyplex nanoparticles. The formulation consisted of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dimyristoyl-sn-glycerol, methoxypolyethylene glycol (DMG-PEG) and linoleic acid. Transferrin or anti-CD45.2 antibody conjugated with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG2000 maleimide) was then post-inserted to the surface of lipoplyplex nanoparticles. The molar ratio of lipids to transferrin was 2000 as previous study[21] and the molar ratio of lipids to anti-CD45.2 antibody was optimized to 10000.

Flow cytometric analysis, sorting of HSCs, CFSE-mediated tracking of cell division, Cobblestone Area Forming-cell assays and Colony-forming assays

Were performed using previously published methods (see also supplemental methods)[22].

In vivo studies

See supplemental methods.

Statistical methods

For clinical correlative statistical analysis on miR-126 expression in primary patient samples see supplemental methods.

For laboratory *in vitro* and *in vivo* experiments, 2-tailed paired Student's *t* tests were performed using GraphPad Prism version 5.0a. P values < .05 were considered significant.

Study approval

See supplemental methods.

Results

Clinical relevance of miR-126 expression in AML

To determine miRs with biologic relevance to LSCs, we identified a miR-expression profile associated with a LSC-specific gene expression signature[1] in AML blasts. One of the most common miRs to be co-expressed with the LSC signature was miR-126. To determine if the variable levels of miR-126 observed in AML blasts had clinical significance, we analyzed miR-126 expression in CN AML patients treated on Alliance/Cancer and Leukemia Group B cytarabine-anthracyclin-based protocols. miR-126 expression levels were higher in younger (<60 years) than older (≥60 years) patients (Figure S1A). However, miR-126 expression levels significantly impacted outcome only in older patients (Figure 1) and not in younger (Figure S1B-D) patients. In older patients, higher miR-126 expression (treated as a continuous variable) was associated with lower complete remission (CR) rate (P=.02) and shorter overall survival (OS) (P=.02) and event-free survival EFS (P=.02) duration (Table 1 and Figure 1A). The significant association of miR-126 levels with clinical (i.e., higher WBC) and molecular features (i.e., higher frequencies of wt *NPM1* and *TET2*, mutated *IDH2* and *RUNX1* and higher expression of *BAALC* and *MN1*) at diagnosis suggested a complex interaction with other biologic events that may concurrently impact prognosis in chemotherapy-treated older CN-AML patients (Supplementary Table S1).

DNA hypermethylation of gene promoter regions is an epigenetic change frequently resulting in gene silencing. Epigenetic regulation of miR-126 expression by DNA methylation has been previously reported in normal and malignant cells[23]. Thus, we also measured miR-126 promoter methylation in the same set of CN-AML patients by Methyl-Cap seq[24]. We found a significantly inverse association between DNA methylation of the miR-126 promoter and miR-126 expression (P=.001). Consistent with this association, we found that lower miR-126 promoter DNA methylation associated with higher miR-126 expression level and correlated with a worse CR rate (P=.01) and shorter OS (P=.003) and EFS (P=.01) duration (see Table 1 and Figure 1B).

Patients with high miR-126 promoter DNA methylation and low miR-126 expression, determined respectively using median values of DNA methylation and expression levels as cutoffs, had a better outcome than the remaining patients (CR P=.08; OS P=.01; EFS P=.03; see Table 1 and Figure 1C).

Only the combined epigenetic/genetic status remained independently associated with outcome (i.e., EFS; P=.01) even after adjusting for other clinical and molecular predictors in a multivariable model (Table 2). Altogether, these results suggest that the miR-126 promoter methylation/expression combined variables seemingly predicted outcome better than miR-126 promoter methylation or expression separately, perhaps reflecting the complexity of miR-126 expression regulation through the combination of both epigenetic and signaling mechanisms[18].

miR-126 expression in AML

In validating data using real time PCR, we showed not only that variable levels of miR-126 expression levels occurred in primary AML blasts, but also that these levels are higher compared to normal bone marrow (BM) mononuclear cells (MNCs) (Figure 2A; see Supplementary Table S2 for patients' molecular features). Previously Lechman et al.[18], showed a preferential expression and functional role for miR-126 in normal HSC and we demonstrated miR-126 expression to be correlated with the LSC-enriched gene expression signature[1], therefore we hypothesized a role for miR-126 in LSC functions in AML. While in normal hematopoiesis HSCs are restricted to the immuno-phenotypically distinct CD34+38-compartment, it has been shown that in AML the stem cells can be found in both CD34+CD38-and CD34+CD38+ subpopulations[25]. To determine which CD34/CD38 compartment was enriched in long-term colony initiating cells (LTC-ICs) in AML patients, we performed limiting dilution assays (LDAs). CD34+CD38- and CD34+CD38+ subpopulations were first isolated by flow sorting and then serial dilutions of each of the two subpopulations were seeded onto irradiated stromal layers. After 6-weeks of culture, cobblestone area forming cells (CAFCs) were scored to determine the presence of LTC-ICs[22]. The LTC-IC-enriched population varied within the CD34/CD38 subpopulations in different patient samples (Figure 2B). Higher miR-126 expression as determined by quantitative RT-PCR was found in the CD34/CD38 LTC-IC-enriched subpopulation for 3 of the 4 analyzed primary AML samples (Figure 2C). In the remaining patient (ptAML-1) no significant difference in miR-126 expression was found between CD34+CD38- and CD34+CD38+ populations. However, a relatively high number of LTC-ICs in both CD34+CD38- and CD34+CD38+ subpopulations was observed in this patient. Conversely, we found that less immature cells (i.e., bulk and CD34-CD38+ blasts) had lower levels of miR-126 expression than CD34+CD38- and CD34+CD38+ blasts (see Figure S2A). Furthermore, when we isolated the progeny of LTC-ICs after 6-weeks of culture we found an increase in miR-126 expression within these cells compared to the CD34+ input cells from day 0 (see figure S2B). Together, these data suggest that increased miR-126 expression characterizes the LTC-IC-enriched CD34/CD38 sub-compartment and may help identify LSC-enriched cell subpopulations within AML patients' samples.

Targeting miR-126 in primary human AML

Having shown the expression of miR-126 is higher in the LTC-IC-enriched cell subpopulations, we hypothesize a role for this miR in LSC self-renewal. To test our hypothesis, we sought to knock-down miR-126 in primary AML cells using Transferrin-conjugated anionic lipopolyplex nanoparticles (Tf-NP) containing antagomiR-126. Recently, we showed that this nanoparticle-based formulation enables achievement of significant intracellular concentration of synthetic miRs or antagomiRs compared to free synthetic miR oligonucleotides in AML blasts expressing high levels of Tf-receptor (CD71)[21]. Since our data and others[25] have reported LSCs to be present in both CD34+CD38- and CD34+CD38+ subpopulations (Figure 2B), we used CD34+ blasts sorted from primary AML patients for these experiments. CD34+ selected cells were treated for 24 hours with Tf-NP containing 200nM of antagomiR-126 (anti-126) or antagomiR-scramble (SCR) and seeded for LTC-IC assays. After six-weeks of co-culture on irradiated stromal layers, CAFCs were scored and LTC-IC frequency determined. Depending on the patient sample,

Tf-NP-antagomiR-126 treated CD34+ cells had a 2.2- to 71.2- fold decrease in LTC-IC frequency compared to Tf-NP-antagomiR-SCR treated controls (Figure 3A). Levels of miR-126 were ∼80% decreased in Tf-NP-antagomiR-126 treated CD34+ blasts compared to Tf-NP-antagomiR-SCR treated controls (P<.01; Figure 3B). Importantly we found no significant differences in apoptosis (Figure 3C) or proliferation (Figure 3D) rates over time in Tf-NP-antagomiR-126 treated CD34+ cells compared to Tf-NP-antagomiR-SCR treated controls.

Tf-NP-antagomiR-126 and Tf-NP-antagomiR-SCR treated CD34+ blasts were also used in colony-forming cell (CFC) assays to assess the impact of miR-126 down-regulation on LP activity. We found no significant differences in the number of CFC in primary Tf-NP-antagomiR-126 treated cells compared to Tf-NP-antagomiR-SCR treated controls scored after 14 days in culture (Figure 3E). To determine if miR-126 knock-down impacted self-renewal capacity, we then harvested primary CFCs and replated them in methylcellulose for additional 14-days in culture. We found significant decreases in the number of CFC in the secondary colonies from Tf-NP-antagomiR-126 treated CD34+ blasts compared with Tf-NP-antagomiR-SCR treated controls (Figure 3F). To determine how miR-126 knock-down impacts different cell states, we also assessed if any changes occurred within the quiescent cell subpopulation. AML blasts were stained with CFSE (Carboxyfluorescein succinimidyl ester) to allow for tracking of viable dividing cells and isolation of undivided CD34+ cells ($CFSE^{max}$/CD34+) after 6 days in liquid culture. Treatment of CD34+ AML blasts with Tf-NP-antagomiR-126 significantly reduced the absolute number of quiescent $CFSE^{max}$/CD34+ cells (P<.05, Figure 3G), which, previously have been reported to be responsible for leukemia re-initiating ability [26, 27]. Altogether these results support a role for miR-126 in leukemic cell self-renewal. The miR down-regulation could lead to unrestrained partial maturation into LP and decrease the quiescent LSC subpopulation while having little effect on survival of proliferating cells.

Targeting miR-126 *in vivo*

Since *in vitro* targeting of miR-126 in CD34+ cells resulted in reduced LTC-IC frequency and the number of quiescent $CFSE^{max}$/CD34+ cells, next we sought to determine whether nanoparticle delivering antagomiR-126 could effectively target LSCs *in vivo*. Briefly, busulfan-conditioned NSG mice were engrafted with human AML primary blasts from patient ptAML-1 (Figure 4A). Eight weeks post primary transplant, mice were treated with Tf-NP-anti-SCR or Tf-NP-anti-126 (n=3 mice per group). Forty-eight hours after the last LNP treatment, BM cells were harvested and transplanted into busulfan-conditioned NSG secondary recipients using two different cell doses (2×10^6 and 5×10^4; n=5 mice per cell dose). We found that mice transplanted with the cells from Tf-NP-antagomiR-126 treated primary human-engrafted mice lived significantly longer than those transplanted with cells from the Tf-NP-antagomiR-SCR treated primary human-engrafted mice ($p<0.05$ for both dose levels; Figure 4B).

Similar results were obtained using our previously established $Mll^{PTD/WT}$ $Flt3^{ITD/ITD}$ double knock-in (dKI) mouse model, which develops an aggressive AML in primary mice, but also leads to death within 6 weeks in secondary BMT experiments. The $Mll^{PTD/WT}$

Flt3$^{ITD/ITD}$ leukemic mouse model has been shown to recapitulate important clinical, cytogenetic and molecular features of the human disease[28]. Similar to the human samples, miR-126 was also found to be increased within the BM Lin-Sca-1+c-kit+ (LSK) compartment, compared to the more mature Lin-Sca-1-c-kit+ (KL) compartment (Figure S3). Using this mouse model allowed us to further characterize the phenotypic consequences of miR-126 knock-down *in vivo*.

We transplanted BM cells from primary dKI AML (CD45.2+) mixed with BM from wild type (WT)-BoyJ (CD45.1+) donors into lethally irradiated WT-BoyJ (CD45.1+) recipients. Since dKI leukemia cells do not express the Tf receptor on their cell surface, we used nanoparticles conjugated to CD45.2 antibody, expressed exclusively on the donor (*Mll*$^{PTD/WT}$ *Flt3*$^{ITD/ITD}$) AML cells to target miR-126 specifically in the leukemia cells. Five weeks post-BM transplantation (BMT), mice were treated once daily for five consecutive days with anti-CD45.2-NP-antagomiR-126 or anti-CD45.2-NP-antagomiR-SCR control (see methods for NP administration). Forty-eight hours after the last dose, BM was harvested from the treated mice (Figure 5A). We confirmed successful targeting of miR-126 *in vivo*, by demonstrating ∼50% decrease in miR-126 expression in BM and spleen from mice treated with anti-CD45.2-NP-antagomiR-126 compared with controls treated with anti-CD45.2-NP-antagomiR-SCR (Figure 5B). The functional consequences of miR-126 down-regulation were assessed by testing the expression of three miR-126 putative targets that have been reported to have a role in cancer: MMP7, a matrix metalloproteinase and known miR-126 target gene[29], CHD7 an epigenetic regulator and possible negative regulator of HSC[30] and JAG1 a Notch ligand, important for normal hematopoiesis[31] (Figure 5C). The expression levels of all three of these putative miR-126 target genes were found significantly increased in the BM cells from anti-CD45.2-antagomiR-126 vs anti-CD45.2-antagomiR-SCR treated primary recipient mice (Figure 5C). Similar results were observed at the protein level (Figure S4). No differences in disease burden or bulk blast viability were noted between the harvests from anti-CD45.2-NP-antagomiR-126 vs anti-CD45.2-NP-antagomiR-SCR treated mice (Figure S5A-B).

To analyze the impact of miR-126 down-regulation on leukemia cell self-renewal activity *in vivo*, we performed secondary transplants using two different cell doses (10^5 and 10^6) of donor BM cells from *Mll*$^{PTD/WT}$ *Flt3*$^{ITD/ITD}$ leukemic mice treated with anti-CD45.2-NP-antagomiR-126 or anti-CD45.2-NP-antagomiR-SCR along with 5.0×10^5 whole bone marrow (WBM) from WT-BoyJ (CD45.1) mice. (Figure 5A) We found significant decreases in the levels of miR-126 in the BM and spleen of anti-CD45.2-NP-antagomiR-126 treated mice compared to anti-CD45.2-NP-antagomiR-SCR controls at the time of harvest (Figure 5B). Interestingly, at this early time point, we found no difference in the frequency of LSK or proliferating cells in the anti-CD45.2-NP-antagomiR-126 BM, suggesting that the difference in miR-126 levels was due to a knock down of the miR expression rather than to a reduction of LSCs/AML cells (Figure S5C-D).

To confirm that anti-CD45.2-NP-antagomiR-126 treatment resulted from a decrease in the number of LSCs resulting in prolonged survival of secondary recipients, we performed LTC-IC assays with BM cells from primary recipients treated with anti-CD45.2-NP-antagomiR-126 or anti-CD45.2-NP-antagomiR-SCR. We found a significant decrease in the

frequency of LTC-ICs in BM cells harvested from anti-CD45.2-NP-antagomiR-126 compared with anti-CD45.2-NP-antagomiR-SCR treated primary recipient mice (P<.001; Figure 5D). Interestingly, when cells from anti-CD45.2-NP-antagomiR-126 or anti-CD45.2-NP-antagomiR-SCR treated primary recipients were used in CFU assays we found that although the colonies were smaller; that there was an increase in the number of CFCs in the primary plating indicating that miR-126 knock-down had no initial, detrimental effect on LPs. However, when these cells were re-plated to assess leukemic cell self-renewal capacity, miR-126 knock-down resulted in a profound decrease in the ability to form colonies in secondary, tertiary, and quaternary re-plating experiments (Figure 5E). These data from *in vivo* treated AML mice are consistent with those obtained with *ex-vivo* treated primary human CD34+ blasts (Figure 3) and indicate the partial exhaustive effect of miR-126 downregulation on LSC.

At a relatively early time point (2-weeks) post-BMT we found a significant decrease in the level of engraftment measured by percentage of chimerism in secondary recipients transplanted with BM cells from anti-CD45.2-NP-antagomiR-126 treated mice compared to recipients transplanted with BM from anti-CD45.2-NP-antimiR-SCR treated primary recipient mice (Figure 5F; P<.01). Following these mice longitudinally, we showed that mice transplanted with 10^6 and 10^5 cells from anti-CD45.2-NP-antagomiR-126 treated primary recipients had respectively a median survival of 46 and 60 days compared with 31 and 35 days of those transplanted with cells from the anti-CD45.2-NP-antagomiR-SCR treated primary recipients (Figure 5G, P<.01 for both cell doses). We performed full pathological analyses of BM or blood from both anti-CD45.2-NP-antagomiR-SCR and anti-CD45.2-NP-antagomiR-126 treated mice, and found no differences in cellular morphology or expression of lineage markers (CD11b, Gr-1, Ter119, CD3, and B220; data not shown). These data demonstrate that *in vivo* knock-down of miR-126 leads to a decrease in functional LSCs.

Although a full toxicology assessment needs to be conducted, we have found no impact of the anti-CD45.2-NP-antagomiR-126 on normal hematopoiesis. When normal CD45.2 C57BL/6J mice with mice were treated with anti-CD45.2-NP-antagomiR-126 or anti-CD45.2-NP-antagomiR-SCR, no statistically significant differences in WBC, BM and spleen cell numbers, or colony-forming ability were seen in mice treated with anti-CD45.2-NP-antagomiR-126 compared to anti-CD45.2-NP-antagomiR-SCR controls (Figure 6 A-C). Furthermore, when we mixed BM from anti-CD45.2-NP-antagomiR-126 or anti-CD45.2-NP-antagomiR-SCR with C57BL/6J-CD45.1 (BoyJ) whole BM at a 1:1 ratio and transplanted into lethally irradiated syngeneic recipient mice (BoyJ), we found no significant differences in hematopoietic reconstitution from CD45.2 cells from the nanoparticle-containing antagomiR-126 treated mice compared to antagomiR-SCR controls (Figure 6D).

Discussion

In AML, LSCs are defined as malignant cells that have acquired stem cell properties such as unlimited self-renewal, quiescence and long-term engraftment/sustained disease maintenance. Because of these acquired features, LSCs constitute a subpopulation of transformed hematopoietic cells capable of recapitulating disease in secondary

transplantation experiments, the gold standard functional assay to verify LSC activity[25, 32]. In addition to coding genes recently we, and others, have demonstrated a leukemogenic and prognostic role for miRs in AML[14-17]. Furthermore we showed previously that increased miR-126 level is associated with enrichment of LSC gene expression in primary patient blasts[1] and more recently that miR-126 has been shown to play a role in regulating normal HSC[33]. Here, we report that variable expression of miR-126 occurs in both younger and older CN-AML patients, albeit, differently from what reported by de Leeuw et al,[33] we observed the clinical impact is age-related since only the older cohort was significantly affected. This may support age-related differences in disease etiology of distinct age-groups. Interestingly, the miR-126 promoter methylation/expression combined variable is a seemingly better predictor of outcome than miR-126 promoter methylation or expression alone, thereby reflecting the complexity of miR-126 expression regulation through the combination of both by epigenetic and signaling mechanisms[18]. This suggests that the altered epigenetic activation of miR-126 expression in leukemia may also lead to concomitant expression of other cancer related genes/miRs via the same epigenetic mechanism, resulting in a more aggressive and therapy-resistant disease phenotype. Altogether these results led us to hypothesize that targeting miR-126 expression may affect LSC activity and confer a therapeutic benefit in older AML patients. We tested this hypothesis by synthesizing a miR-126 antagomiR formulated in targeting (antigen or antibody-conjugated) NPs[21]. This approach enabled us not only to gain biologic insight into the role of miR-126 in AML LSCs, but also to lay the foundation for a future miR-based therapeutic approach for targeting this minute, but biologically and clinically relevant, subpopulation of leukemia cells.

Lechman et al.,[18] reported miR-126 to be important for the regulation of normal HSC and HPC, and demonstrated potentially different functions for miR-126 in these cell compartments. Using lentiviral knock-down ‘sponge’ vectors to inhibit miR-126 function, they found that miR-126 knock-down resulted in an increase in HSC proliferation without exhaustion, *In vivo* they noted a long-term reduced hematopoietic activity supported by decreased output of myeloid and B cells. In contrast, when miR-126 was overexpressed in normal HSC (CD34+CD38-CD90+) the number of quiescent cells increased [18]. Therefore, miR-126 seemingly plays a regulatory role in hematopoiesis by balancing HSC proliferative/differentiation activities. Since increased miR-126 in normal HSCs resulted in increased numbers of quiescent cells, we hypothesized that miR-126 may also have a pivotal role in AML by regulating the fractions of LSCs that remain quiescent and those that proliferate. Therefore, decreased miR-126, may deplete the reservoir of quiescent LSCs and reduce leukemia growth. This hypothesis was supported by our results showing decreases in the number of quiescent $CFSE^{max}$/CD34+ leukemia cells after *in vitro* treatment with antagomiR-126 and the longer survival observed in mice subjected to secondary transplant with marrow cells from antagomiR-126 treated primary leukemic donors.

The biology of LSCs has been shown to be distinct from that of the bulk blasts genetically, epigenetically, and functionally. Although LSCs only comprise a very small population within AML, they most likely have a profound impact on the clinical presentation and biology of the disease and need to be eradicated in order to achieve cure in AML patients.

However, efficient therapeutic targeting of the small LSC subpopulation *in vivo* has been challenging. We now show this limitation may be overcome by using antagomiR-126 delivery via novel specific antigen (Tf) or antibody (anti-CD45.2)-conjugated anionic lipopolyplex nanoparticles. As we previously reported, the chemical and physical characteristics of this formulation allow for bypassing hepatic uptake and ultimately a better delivery of the nanoparticle compound to hematopoietic organs without any evidence of toxicity[21]. In our studies we showed that NP delivery of antagomiR-126 can be achieved in rare AML subpopulations such as LSCs *in vivo*, and was not toxic to normal hematopoietic functions since normal BM treated with anti-CD45.2-NP-antagomiR-126 showed no hematopoietic deficits. The activity of the NP-antagomiR-126 seems quite rapid, suggesting that even a relatively brief perturbation of miR-126 expression may permanently impact LSC function and lay the basis for potentially new therapeutic approaches. Importantly, although the antagomiR-126 was not modified with any fluorescent reporter to allow for a direct quantifiable assessment of oligo uptake in vivo, we are confident that robust intracellular oligos' (antagomiR-126 and antagomiR-SCR) concentrations were sufficient that after only a few doses of nanoparticles, we were able to demonstrate significant knock-down of miR-126 *in vivo*, increase the expression of miR-126 target genes and decrease LSC frequency in BM from anti-126-treated mice compared to anti-SCR-treated controls. This resulted in a delay of disease development and increased survival of leukemic mice in secondary transplants performed with equal numbers of viable mononuclear BM cells from anti-126-treated and anti-SCR-treated primary donor cells. Although the brief treatment with NP-antagomiR-126 had little impact on disease burden in the primary mice since the percent of human primary cells in the BM of anti-126 treated mice was not significantly different than the anti-SCR treated mice, 91.5±2.8% vs. 90.7±2.6% respectively (NS, n=3 mice per group).we were able to achieve a significant impact on survival in secondary recipients. These data suggest that the effect of antagomiR-126 may be most beneficial in residual LCS-enriched population cells similar to those that persist after treatment and likely drive disease relapse in chemotherapy-treated patients. Thus, it is reasonable to envision that combination of miR-126 inhibition and cytoreductive chemotherapy to target both proliferative “bulk” blasts and LSCs, may be a compelling strategy to eradicate the disease and prevent leukemia relapse in AML patients. Of course, delivery of antimiR-based therapeutics may need to be further refined for use in humans by optimizing NP packaging or considering emerging alternative formulations[34].

In conclusion, we report the biological and preclinical relevance of targeting miR-126 in AML. We demonstrate the feasibility of miR-126 knock-down *in vivo*, interfering with LSC self-renewal utilizing a novel, cutting-edge antibody-conjugated nanoparticle approach. Recently *in vivo* targeting of miR-196b was also reported to successfully eradicate LSCs. However, the relevance of this miR was seemingly limited to AML blasts harboring MLL translocations[35], while miR-126 has been found highly expressed in two of the most frequent cytogenetic subtypes of AML i.e., CN[1] and core binding factor[33] that together represent approximately 60% of all the AML cases. Therefore, the therapeutic targeting of miR-126 using NP-antagomiR-126 is potentially applicable to a broader group of AML patients. While the therapeutic development of NP-antagomiR-126 is beyond the scope of the present work and precise pharmacokinetic/pharmacodynamics modeling to define the

optimal dose and schedule of NP-antagomiR-126 to be used *in vivo* is needed, it seems reasonable to predict that combinations of NP-antagomiR-126 with cell cycle-dependent chemotherapeutics may be needed to completely eradiate the disease to achieve both effective cytoreduction of bulk blasts and cycling LPs, and LSC elimination, thus resulting in complete eradication of the disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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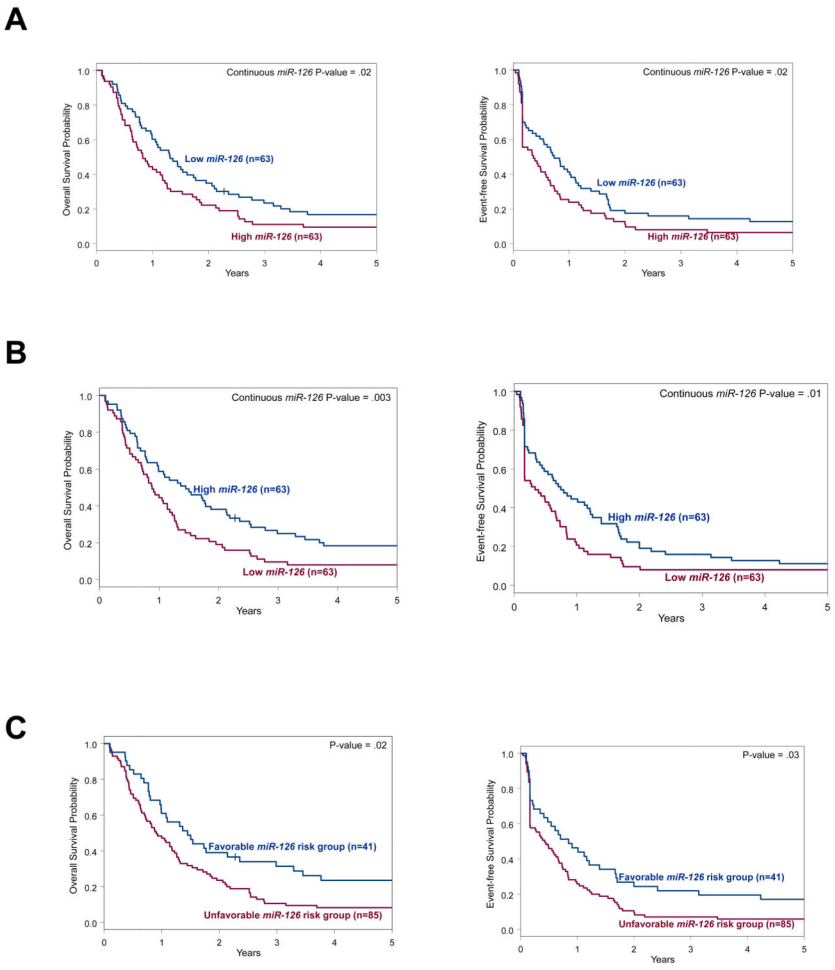
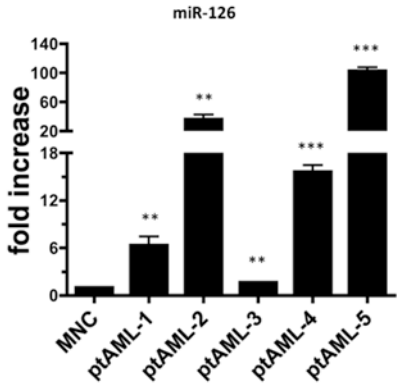


Figure 1. Prognostic impact of miR-126 expression and promoter DNA methylation in older Cytogenetically Normal AML patients

A. Impact of miR-126 expression levels on Overall Survival (OS) and Even-free Survival (EFS); **B.** Impact of miR-126 promoter DNA methylation levels on OS and EFS. For A and B miR-126 expression and DNA methylation are considered continuous variables; patients were dichotomized to the median expression and DNA methylation level values for the purpose of graphical representation. **C**. OS and EFS according to the miR-126 promoter methylation/expression dual status in older *de novo* CN-AML. The favorable risk group comprised patients with miR-126 low expression/high methylation; the unfavorable risk group comprised the remaining patients (high expression/low methylation, high expression/high methylation, low expression/low methylation). High and low expression and methylation was defined by using median values as cut-offs.

A



B

AML Patient Sample ID	CD34+ CD38- Frequency 1 LSC per n cells (95% CI)	CD34+ CD38+ Frequency 1 LSC per n cells (95% CI)
ptAML-1	$5.8x10^2$ ($3.1x10^2$ - $1.9x10^3$)	$2.4x10^1$ ($1.3x10^1$- $4.6x10^1$)
ptAML-2	$9.1x10^3$ ($4.9x10^3$ - 1.7^4)	$1.9x10^3$ ($1.1x10^3$- $3.5x10^3$)
ptAML-3	$4.1x10^3$ ($1.9x10^3$ - $8.7x10^3$)	**1< LSC per x $5.0x10^4$ cells
ptAML-4	$6.9x10^2$ ($3.0x10^2$ – $1.6x10^3$)	$3.2x10^5$ ($4.7x10^4$ – $2.3x10^6$)

C

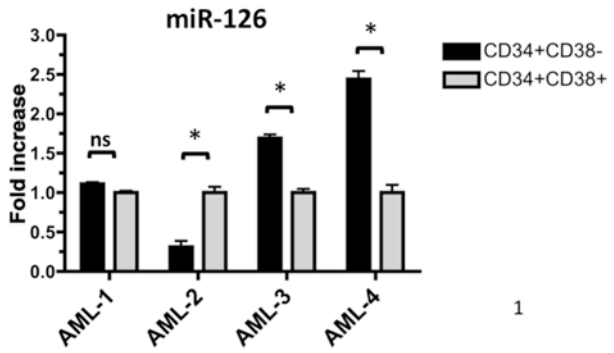


Figure 2. LTC-IC frequency and miR-126 expression in AML

A. Normal BM mononuclear cells (MNCs) from normal donors (n=3) were pooled and compared to AML leukopheresis samples (n=5). miR-126 levels were measured in AML samples by real time RT-PCR and the results were normalized to an endogenous reference (U44 small RNA) and reported relatively to the levels in normal MNC. Data are shown as mean ± s.d. (**P<.01, ***P<.001). **B.** Frequency of **LTC-ICs** in sorted CD34+/CD38- and CD34+/CD38+ AML subpopulations. Sorted samples were co-cultured for 6 weeks on irradiated stromal layers in limiting dilution conditions. **C.** Real-time RT-PCR of miR-126

in sorted CD34+/CD38- and CD34+/CD38+ AML samples. miR-126 expression was normalized to an endogenous reference (RNU44 small RNA) and expressed for the CD34+/CD38- population relatively to the CD34+CD38+ population (mean ± s.d.), ns= not significant, * P<.05).

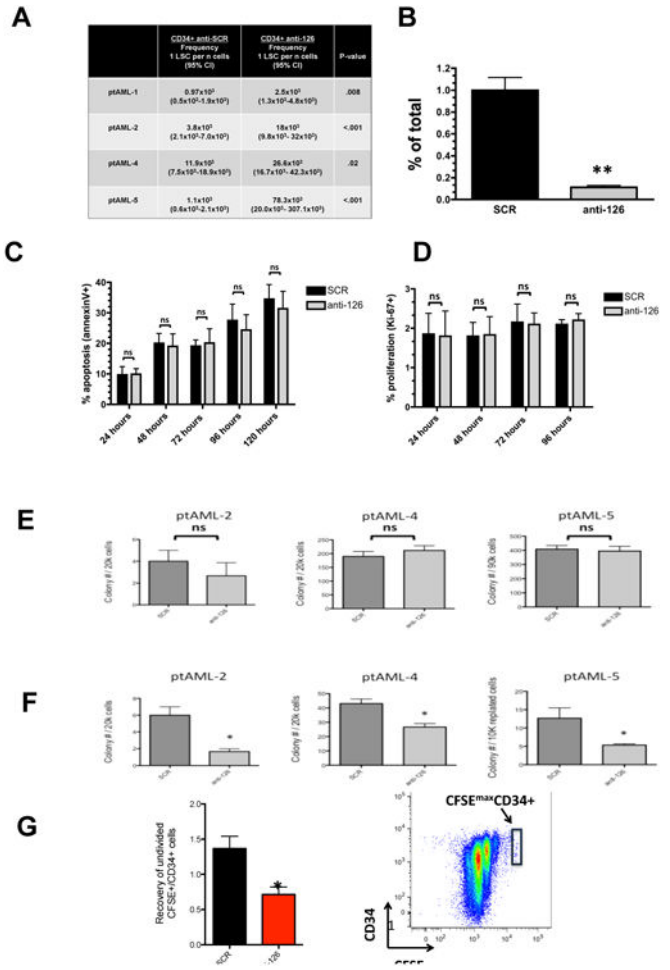


Figure 3. Effect of nanoparticle-mediated miR-126 knock-down on LTC-IC survival and self-renewal

A. LTC-IC frequency in Tf-NP-antagomiR-SCR or Tf-NP-antagomiR-126-treated (24 hours) CD34+ cells from AML patient samples. Sorted patient samples were co-cultured for 6 weeks on irradiated stromal layers in limiting dilution conditions. **B.** miR-126 expression in CD34+ cells from AML patient samples at 48 hours after treatment with Tf-NP-antagomiR-SCR or Tf-NP-antagomiR-126. **C.** Percent of cells undergoing apoptosis measured using AnnexinV staining and flow cytometry at indicated time points. (mean ± s.d., n=3, ns=not significant). D. Percent of cells undergoing proliferation using Ki-67 antibody and flow cytometry at indicated time points. (mean ± s.d., n=3, ns=not significant). **E.** Primary CFU assays of CD34+ cells from AML patient samples (n=3) pre-treated with Tf-NP-antagomiR-SCR or Tf-NP-antagomiR-126 for 24 hours. **F.** Re-plating assays of cells harvested from the primary CFUs. (mean ± s.d., ns=not significant, * P<.05). **G.** Recovery of quiescent CFSEmax/CD34+ cells after a 6 day culture of CFSE-labeled CD34+ AML cells treated (at 72 hours) with Tf-NP-antagomiR-SCR or Tf-NP-antagomiR-126 (mean ± s.e.m., n=3, * P<.05).

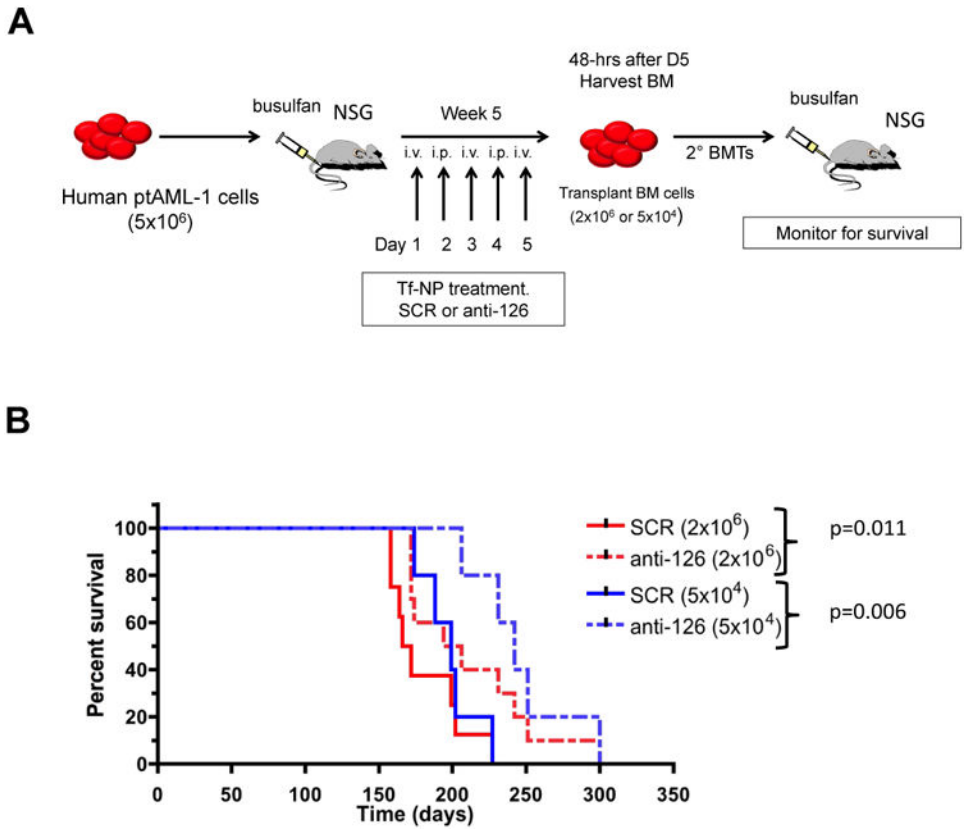


Figure 4.

Effect of miR-126 knock-down in primary human AML patient sample *in vivo*. A. Schematic representation of the experimental design (see also Methods) antagomiR-scramble (SCR), antagomiR-126 (anti-126). B. Survival curve of secondary recipients engrafted with indicated cell dose from human primary AML patient sample (ptAML-1) (n=5 recipients per group).

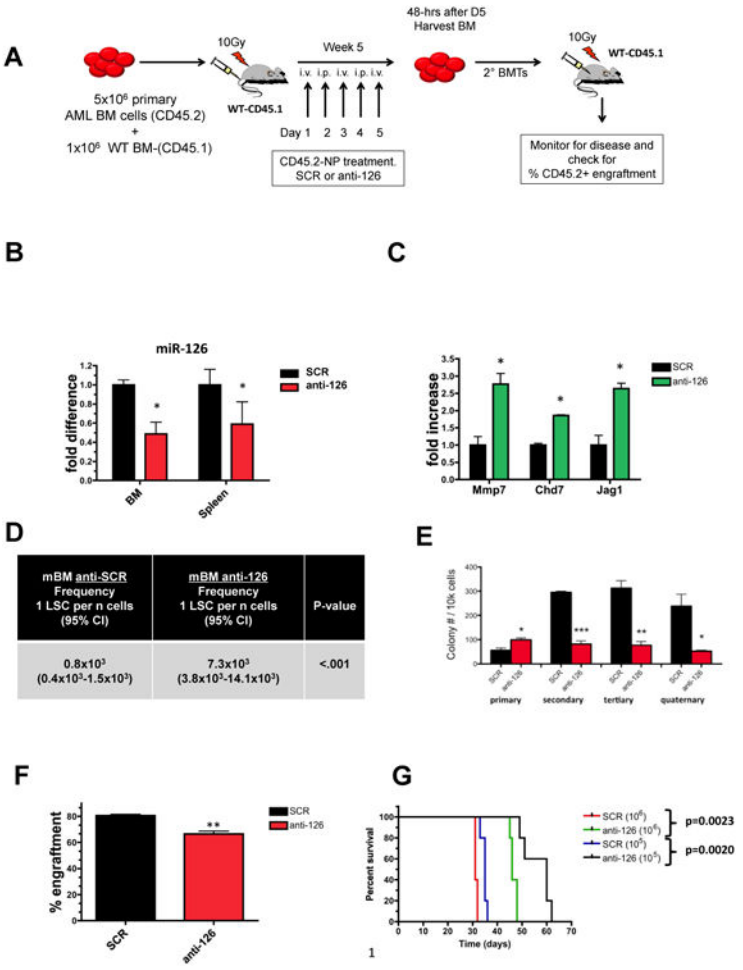


Figure 5. Effect of miR-126 knock-down in primary mouse AML sample *in vivo*

A. Schematic representation of the experimental design (see also Methods) antagomiR-scramble (SCR), antagomiR-126 (anti-126). **B.** miR-126 real time RT-PCR on sorted CD45.2+ donor leukemia cells 48 hours after final *in vivo* treatment with anti-CD45.1-NP-antagomiR-SCR or anti-CD45.1-NP-antagomiR-126. (mean ± s.d., *P<.05). **C.** Real time RT-PCR of putative miR-126 target genes 48 hours after the last dose of anti-CD45.2-NP-antagomiR-SCR or anti-CD45.2-NP-antagomiR-126 treatment. (mean ± s.d.) (*P<.05, **P<.01, ***P<.001). **D.** LTC-IC of mouse bone marrow from anti-CD45.2-NP-antagomiR-SCR or anti-CD45.2-NP-antagomiR-126 treated primary transplant mice (mean ± s.d., n=3 mice per group, P<.001). **E.** Primary CFU and re-plating assays of BM cells from anti-CD45.2-NP-antagomiR-SCR or anti-CD45.2-NP-antagomiR-126 treated mice (mean ± s.d.) (n=3 mice treated per group, pooled for each assay, *P<.05, **P<.01, ***P<.001). **F.** Donor chimerism in peripheral blood (n=5 mice per group) 2 weeks post-BMT from mice transplanted with 1×10^6 leukemic cells (CD45.2+) harvested from mice treated with anti-CD45.2-NP-antagomiR-SCR or anti-CD45.2-NP-antagomiR-126, and co-transplanted with 5.0×10^5 normal WBM (CD45.1+). (mean ± s.d., **P<.01). **G**. Survival of secondary recipients receiving either 10^5 or 10^6 cells from anti-CD45.2-NP-antagomiR-SCR or anti-

CD45.2-NP-antagomiR-126 treated primary transplanted mice, co-transplanted with 5×10^5 (CD45.1+) normal WBM (n=5 mice per group).

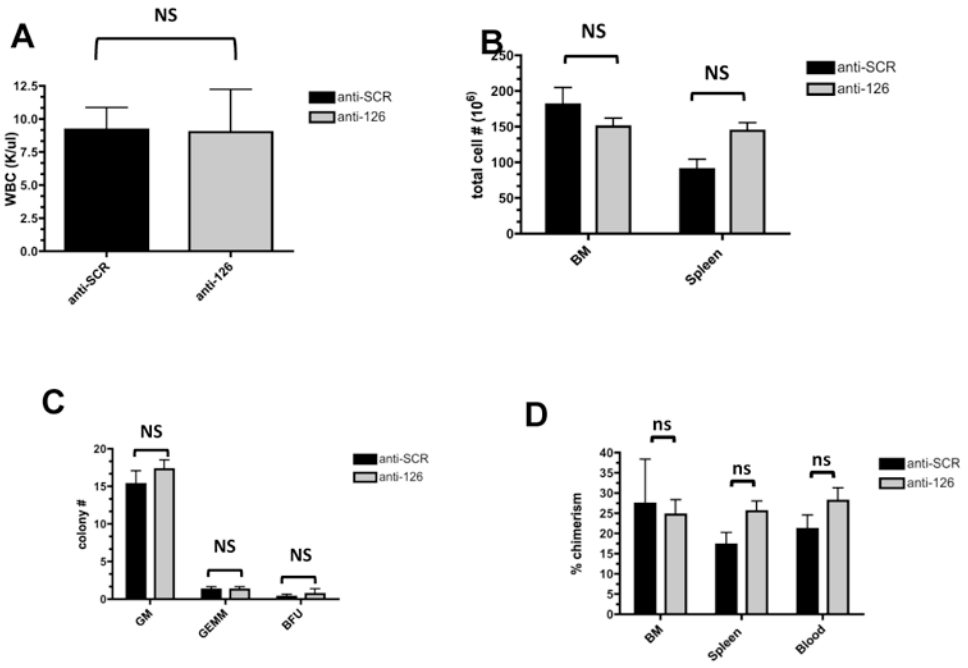


Figure 6. Effect of NP-antagomiR-126 on normal hematopoiesis *in vivo*

A: Wild type mice (CD45.2-C57Bl/6J) (n=3 mice per group) were treated with the identical treatment regimen outlined in Fig.5A and the effect on normal hematopoiesis was assessed in peripheral blood by A. WBC (mean ± s.d., n=3 mice per group, p=ns). **B.** Spleen total cell numbers (mean ± s.d., p=ns). **C.** Primary CFU assays using WBM from anti-CD45.2-NP-antagomiR-SCR or anti- CD45.2-NP-antagomiR-126 treated mice, and then were scored 14-days later for colony type, GM=granulocyte/macrophage, GEMM=granulocyte/erythroid/macrophage/megakaryocyte, BFU=burst-forming unit-erythroid (mean ± s.d., n=3, p=ns). **D**. Donor chimerism in peripheral blood (n=5 mice per group) 8 weeks post-BMT from mice transplanted with 2×10^6 (CD45.2-C57Bl/6J) BM cells harvested from mice treated with anti-CD45.2-NP-antagomiR-SCR or anti-CD45.2-NP-antagomiR-126, and co-transplanted with $2.\times10^6$ normal WBM (CD45.1+) (mean ± s.d., n=5 recipients per group, p=ns).

Table 1
Prognostic impact of miR-126 expression and promoter DNA methylation

1.A miR-126 expression

n= 126	Low *mir-126* (n=63)	High *mir-126* (n=63)	p§
CR, no. (%)	46 (73)	35 (56)	.02
Overall Survival (OS)			.02
Median (years)	1.3	0.8	
%Alive at 3 years	23 (14-35)	11 (5-20)	
%Alive at 5 years	17 (9-27)	10 (4-18)	
Event-Free Survival (EFS)			.02
Median (years)	0.7	0.4	
%Event-free at 3 years	16 (8-26)	8 (3-16)	
%Event-free at 5 years	13 (6-22)	6 (2-14)	

B Promoter DNA methylation of miR-126

n= 126	Low *methylation* (n=63)	High *methylation* (n=63)	p§
CR, no. (%)	35 (56)	46 (73)	.01
Overall Survival (OS)			.003
Median (years)	0.9	1.4	
%Alive at 3 years	10 (4-18)	25 (15-36)	
%Alive at 5 years	8 (3-16)	18 (10-29)	
Event-Free Survival (EFS)			.01
Median (years)	0.3	0.7	
%Event-free at 3 years	8 (3-16)	16 (8-26)	
%Event-free at 5 years	8 (3-16)	11 (5-20)	

C Combination of miR-126 Promoter DNA methylation and expression

n= 126	Favorable risk group (n=41)	Unfavorable risk group (n=85)	p†
CR, no. (%)	31 (76)	50 (59)	.08
Overall Survival (OS)			.02
Median (years)	1.4	0.9	
%Alive at 3 years	31 (18-46)	11 (5-18)	
%Alive at 5 years	24 (12-37)	8 (4-15)	
Event-Free Survival (EFS)			.03
Median (years)	0.8	0.4	
%Event-free at 3 years	22 (11-35)	7 (3-14)	
%Event-free at 5 years	17 (8-30)	6 (2-12)	

§P-values are for the continuous variables. For CR, logistic regression was used, for time to event variables Cox regression models were run.

[†]P-values for categorical variables are from Fisher's exact test, p-values for time to event variables are from the log-rank test.

Table 2
Multivariable analysis for EFS in older CN-AML

Variable		P	Hazard Ratio (95% CI)
*miR-126risk group	Favorable vs Unfavorable	.01	0.53 (0.32, 0.86)
**_BAALC_	High vs Low	.006	1.90 (1.20, 3.00)
**miR-155	High vs Low	.03	1.65 (1.06, 2.56)
Platelets	Continuous, 50-unit increase	.008	1.16 (1.04, 1.30)
WBC	Continuous, 50-unit increase	.02	1.20 (1.03, 1.41)

* The favorable miR-126risk group consists of patients with low *miR-126* expression and high *miR-126* methylation. The unfavorable miR-126 risk group consists of the rest of the patients.

** High and low *BAALC* and miR-155 expressers were identified using a median value as the cutoff. These genes were measured by RT-PCR or nanostring assays as previously reported[7].