Micro-ribonucleic acid-155 is a direct target of Meis1, but not a driver in acute myeloid leukemia

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ABSTRACT

Micro-ribonucleic acid-155 (miR-155) is one of the first described oncogenic miRNAs. Although multiple direct targets of miR-155 have been identified, it is not clear how it contributes to the pathogenesis of acute myeloid leukemia. We found miR-155 to be a direct target of Meis1 in murine Hoxa9/Meis1 induced acute myeloid leukemia. The additional overexpression of miR-155 accelerated the formation of acute myeloid leukemia in Hoxa9 as well as in Hoxa9/Meis1 cells *in vivo*. However, in the absence or following the removal of miR-155, leukemia onset and progression were unaffected. Although miR-155 accelerated growth and homing in addition to impairing differentiation, our data underscore the pathophysiological relevance of miR-155 as an accelerator rather than a driver of leukemogenesis. This further highlights the complexity of the oncogenic program of Meis1 to compensate for the loss of a potent oncogene such as miR-155. These findings are highly relevant to current and developing approaches for targeting miR-155 in acute myeloid leukemia.

Introduction

Leukemogenesis is a complex multistep process that impacts differentiation, proliferation and self-renewal. Large profiling approaches such as the Cancer Genome Project defined mutations in genes such as *FLT3, NPM1, DMNT3A* and *NRAS* as driver mutations in acute myeloid leukemia (AML).[1,2] Mutations in miRNAs, a class of short non-coding RNAs, are rare, but quantitative expression studies have led to a better understanding of how deregulation of miRNAs associates with genetic subgroups of AML.[1,3] However, only functional studies allow the elucidation of the potential of miRNAs as drivers in leukemia and as therapeutic targets. It is generally assumed that a single miRNA has hundreds of putative targets and can therefore simultaneously affect multiple pathways and processes. In hematopoiesis, specific miRNA expression patterns[4] maintain a fine balance between hematopoietic stem and progenitor cell (HSPC) self-renewal and differentiation[5] as well as between normal and malignant hematopoiesis.[6] Therefore, the targeting of miRNAs holds promise for advancing targeted cancer therapies of currently undruggable genetic translocations and pathways.

One of the first described oncogenic miRNAs, miR-155, was originally identi-

fied to be overexpressed in both lymphomas and in AML.[7-9] However, the roles of miR-155 in leukemogenesis and hematopoiesis are more complex, as it impacts inflammatory processes, B-cell and T-cell function in addition to myeloid development.[8,10-13] We have recently shown that miR-155 functions in HSPC mobilization,[14] suggesting that miR-155 bears a more complex role in stem cell physiology than previously assumed. We also reported that miR-155 levels are correlated with *MLL* translocations, an AML subtype characterized by high *HOX* gene and *MEIS1* levels.[15]

In AML, transcript levels of *HOXA9* are highly correlated with poor prognosis,[16] and engineered overexpression of Hox proteins in hematopoietic cells results in long latency leukemia in mice, indicating that collaborating genetic events are required for full leukemic transformation.[17,18] The HOX cofactor, Meis1, is rate-limiting for MLL-rearranged AML and has been identified as collaborating with HOX proteins and HOX fusions (NUP98-HOX) to induce a rapid disease onset of AML in mice.[19-21] With the aim of identifying leukemia-contributing miRNAs and defining their roles in leukemogenesis, we sought to build a clinically relevant model system for AML. Using a Hoxa9 and Meis1 murine AML progression model,[22] together with findings in human AML, herein we have identified deregulated miRNAs downstream of Hoxa9 and Meis1, and have further characterized the role of miR-155 in AML development as well as its potential as a therapeutic target both *in vitro* and *in vivo*.

Methods

Retroviral and Lentiviral constructs

Murine stem cell virus (MSCV)-based retroviral vectors carrying expression cassettes consisting of *Hoxa9*[23] *Meis1*[24] *ΔHDMeis1*[17] and miR-155[8] have been described previously. The generation of recombinant retrovirus-producing GP-E86 cells was performed as previously described.[25] The lentiviral miR-155 sponge vector BdLV.155pT and the scrambled control vector BdLV.Ctrl have been formerly elucidated.[26]

Generation of transduced murine bone marrow (BM) cells and transplantation assays

A detailed description of the generation of cell lines and cell culture as well as proliferation and clonogenic assay experiments are described in the *Online Supplementary Methods*. All animal experiments were approved by the state government of Gothenburg, Sweden and Tuebingen, Germany. Transplantation assays were performed as previously described.[17,25]

Detailed engraftment analysis, sick mice workup, limiting dilution and homing assay are also described in the *Online Supplementary Methods.*

Chromatin immunoprecipitation and sequencing (ChIP-seq)

ChIP-seq of the Hoxa9/Meis1 cell line was performed using standard operating procedures for ChIP-seq library construction as previously described.[27] Details of antibodies that were used are described in the *Online Supplementary Methods*. Enriched Meis1 binding regions on chromosome 16 in Hoxa9/Meis1 cells are summarized in *Online Supplementary Table S1*. Identified peaks for the determined histone modifications on chromosome 16 are listed in *Online Supplementary Table S2* for Hoxa9/ctrl cells and *Online Supplementary Table S3* for Hoxa9/Meis1 cells.

MiRNA and messenger (m)RNA expression arrays

RNA for the array analysis was prepared from independently generated cell lines three to four weeks post transduction, expressing Hoxa9/ctrl (n=9), Hoxa9/Meis1 (n=4), Hoxa9/ΔHDMeis1 (n=4), Hoxa9/Meis1$^{155-/-}$ (n=3) or Hoxa9/miR-155 (n=3). A detailed data analysis is described in the *Online Supplementary Methods*. The Gene Expression Omnibus (GEO) accession numbers for miRNA array and mRNA array of Hoxa9/ctrl, Hoxa9/Meis1 and Hoxa9/ΔHDMeis1 are GSE74566 and GSE75272, respectively. Exon array data for Hoxa9/ctrl, Hoxa9/miR-155 and Hoxa9/Meis1$^{155-/-}$ are available at GSE76113.

Human samples from healthy donors and AML patients

All samples were collected according to protocols approved by the Ethics Committee of the University Hospital of Ulm. All probands gave informed consent for genetic analysis according to the Declaration of Helsinki. Detailed descriptions of the patient samples used in this study are provided in the *Online Supplementary Methods.*

Real-time quantitative polymerase chain reaction (RT-qPCR)

Detailed RT-qPCR experiments and primers are described in the *Online Supplementary Methods.*

Flow cytometric analysis and fluorescence activated cell sorting (FACS)

Immunophenotype analysis was performed on either a LSRFortessa™ cell analyzer or FACSAria II and cell sorting on a FACSAria II or FACSAria III sorter (Becton Dickinson Biosciences). Antibodies used for immunophenotype determination and subpopulation sorting are described in the *Online Supplementary Methods.*

Microfluidics analysis

Mononuclear cells prepared from diagnostic peripheral blood (PB) of 17 newly diagnosed cytogenetically normal (CN)-AML (FLT-internal tandem duplication (ITD) pos, n=9) patient samples were sorted into CD34$^+$CD38$^-$, CD34$^+$ and myeloid-enriched subpopulations and processed as previously described. Antibodies used for immunophenotype determination and cycle threshold (Ct) value calculations are described in the *Online Supplementary Methods*. Samples were collected at the British Columbia Cancer Agency (BCCA, Vancouver, BC, Canada) according to protocols approved by the Ethics Committee of the BCCA.

Western blot analysis

Protein extraction and immunoblotting was performed *via* standard procedures using the following antibodies per manufacturer's instructions: anti-CD13 (ANPEP, clone EPR4058) (Abcam), anti-JARID2 (Novus), and anti-β-actin (clone AC-15) (Sigma-Aldrich).

Statistics

Pairwise comparisons were performed using the Mann-Whitney U test, unless otherwise specified. The Kaplan-Meier method with log-rank test was used to compare differences between survival curves. Spearman correlation was used for tests of relationships. Leukemia-initiating cell (LIC) frequencies were calculated with L-Calc™ Software Version 1.1 (STEMCELL Technologies). Unless otherwise indicated, data are expressed as mean ± standard error of the mean (s.e.m.). A *P*-value of less than 0.05 was defined as statistically significant; statistical significance is shown as $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

Results

MiR-155 is a direct target of Meis1

To identify miRNAs relevant for transforming HSPCs into AML cells, we compared the miRNA transcriptome of pre-leukemic cells overexpressing Hoxa9 and leukemic cells co-overexpressing Hoxa9 and Meis1.[22] Murine BM cells were transduced to generate cell lines overexpressing Hoxa9 alone (with an empty vector control, Hoxa9/ctrl, n=9), Hoxa9 together with a mutant and inactive Meis1 lacking the homeodomain (Hoxa9/ΔHDMeis1, n=4) or wild-type (wt) Meis1 (Hoxa9/Meis1, n=4).[17,28]

Analysis of the miRNA transcriptome identified 16 significantly deregulated miRNAs (Table 1). Of these, miR-155-5p (henceforth referred to as miR-155) was the most significantly upregulated miRNA in Hoxa9/Meis1 cells compared to the Hoxa9/ctrl and Hoxa9/ΔHDMeis1 cells. The upregulation of miR-155 was validated in independently generated Hoxa9/Meis1 cells (Figure 1A). Meis1 or Hoxa9 overexpression alone did not result in the upregulation of miR-155 compared to BM cells transduced with an empty control vector, indicating that miR-155 upregulation requires co-expression of Hoxa9 and Meis1 (Figure 1A). In line with this observation, the host gene of miR-155, *miR-155hg (Bic),* was also more highly expressed in Hoxa9/Meis1 compared to Hoxa9/ctrl cells, but not in cells that ectopically expressed either Meis1 or Hoxa9 only (Figure 1B). We further validated the interaction of Meis1 with the *miR-155hg/miR-155* locus in Hoxa9/Meis1 cells and characterized epigenetic changes (H3K4me, H3K27ac, H2K4me1, H3K36me3) by ChIP-seq. Our data demonstrate that Meis1 binds to a region approximately 4kb upstream of *miR-155hg* (Figure 1C and *Online Supplementary Figure S1A*), accompanied by a marked increase in H3K27ac. We further detected an increase in H3K4me3 at the promoter and 5' region of *miR-155hg* as well as enrichment of H3K36me3 along the gene body in Hoxa9/Meis1 cells (Figure 1C). H3K27ac and H3K4me3 levels in Hoxa9/ΔHDMeis1 were similar to that of Hoxa9/ctrl cells (*data not shown*). The direct binding of Meis1 to a putative enhancer element and the enrichment of activating epigenetic marks is in line with increased transcript levels of *miR-155hg* and miR-155 in Hoxa9/Meis1 cells.

MiR-155 augments the leukemogenic potential of Hoxa9

Based on the upregulation of miR-155 in leukemic Hoxa9/Meis1 cells and its known oncogenic potency,[29] we hypothesized that miR-155 may fully or partially account for the leukemogenic properties of Meis1 when co-expressed with Hoxa9. In addition, we tested the transforming potential of miR-155 alone by transduction of murine BM with miR-155 or an empty control vector. The overexpression levels of miR-155 in Hoxa9/miR-155 cells and cells overexpressing miR-155 alone are shown in *Online Supplementary Figure S1B*. *In vitro* analysis of cell lines overexpressing Hoxa9/miR-155 showed no difference in proliferation when compared to Hoxa9/ctrl, whereas Hoxa9/Meis1 cells grew significantly faster in liquid culture compared to Hoxa9/ctrl (*Online Supplementary Figure S1C*). The colony forming capacity of Hoxa9/miR-155 was significantly elevated to a level similar to that found in Hoxa9/Meis1 cells when compared to Hoxa9/ctrl (*Online Supplementary Figure S1D*). The immunophenotype

Table 1. Differentially expressed miRNAs between Hoxa9/Meis1 and Hoxa9/ctrl or Hoxa9/ΔHDMeis1.

miRNAs	*P* value	Fold change Hoxa9/Meis1 *vs.* Hoxa9/Meis1	Fold change Hoxa9/Meis1 *vs.* Hoxa9/ΔHDMeis1
miR-155-5p	9.08E-03	2.4	3.5
miR-708-5p	1.04E-02	6.7	6.4
miR-30a-3p	1.39E-02	5.2	4.5
miR-182-5p	1.77E-02	2.1	2.0
miR-449a-5p	2.18E-02	3.9	2.4
miR-155-3p	3.32E-02	2.8	3.0
miR-30c-2-3p	3.32E-02	2.3	2.5
miR-30a-5p	3.77E-02	2.0	2.1
miR-183-5p	3.92E-02	4.0	2.7
miR-501-5p	9.41E-03	-2.3	-2.0
miR-99b-5p	9.41E-03	-5.2	-4.8
miR-146a-5p	1.83E-02	-4.8	-6.1
miR-466b-3p	1.83E-02	-2.0	-3.9
miR-125a-5p	3.39E-02	-3.1	-3.9
miR-511-3p	4.29E-02	-4.2	-2.2
miR-466e-3p	4.89E-02	-2.0	-3.9

of Hoxa9/miR-155 was also comparable to that of Hoxa9/Meis1 cells with significantly more c-kit-expressing cells and lower numbers of the more mature cells expressing Mac-1 and Gr-1 (*Online Supplementary Figure S1E*). MiR-155 overexpressing BM cells showed increased proliferation and colony formation *in vitro* (*Online Supplementary Figure S1C,S1D*), supporting the idea that miR-155 may possess transforming potential by increasing self-renewal capacity. Based on the recent report by Narayan *et al.*,[30] we investigated the kinetics of miR-155 upregulation and of a possible selection process during Hoxa9/Meis1-mediated transformation by quantification of miR-155 in an *in vitro* time course experiment. MiR-155 upregulation was stable over time (Figure 1D), demonstrating that in our model miR-155 expression is not associated with differentiation and endogenous selection in Hoxa9/Meis1 cells.

In vivo, mice transplanted with Hoxa9/miR-155 showed higher engraftment levels compared to Hoxa9/ctrl, but to a lower extent than mice transplanted with Hoxa9/Meis1 cells (Figure 2A). Overexpression of miR-155 alone did not enhance engraftment, and transplanted mice remained healthy (Figure 2A,B). The Hoxa9/miR-155 mice succumbed to leukemia with a median latency of 82 days (range: 32-153 days), corresponding to approximately twice the median latency of mice transplanted with Hoxa9/Meis1 (36 days, range: 28-62 days) and half the median latency of mice transplanted with Hoxa9/ctrl cells (162 days, range: 106-206 days) (Figure 2B). Mice transplanted with Hoxa9/miR-155 cells developed an acute myelomonocytic leukemia, based on the Bethesda criteria for non-lymphoid neoplasias[31] (Figure 2C). Similar to Hoxa9/Meis1 induced leukemias that also show a myelomonocytic phenotype, mice transplanted with Hoxa9/miR-155 cells exhibited hepatomegaly and splenomegaly comparable to Hoxa9/ctrl (*Online Supplementary Figure S2A*). Blood cell counts of Hoxa9/miR-155 mice showed increased leukocytosis

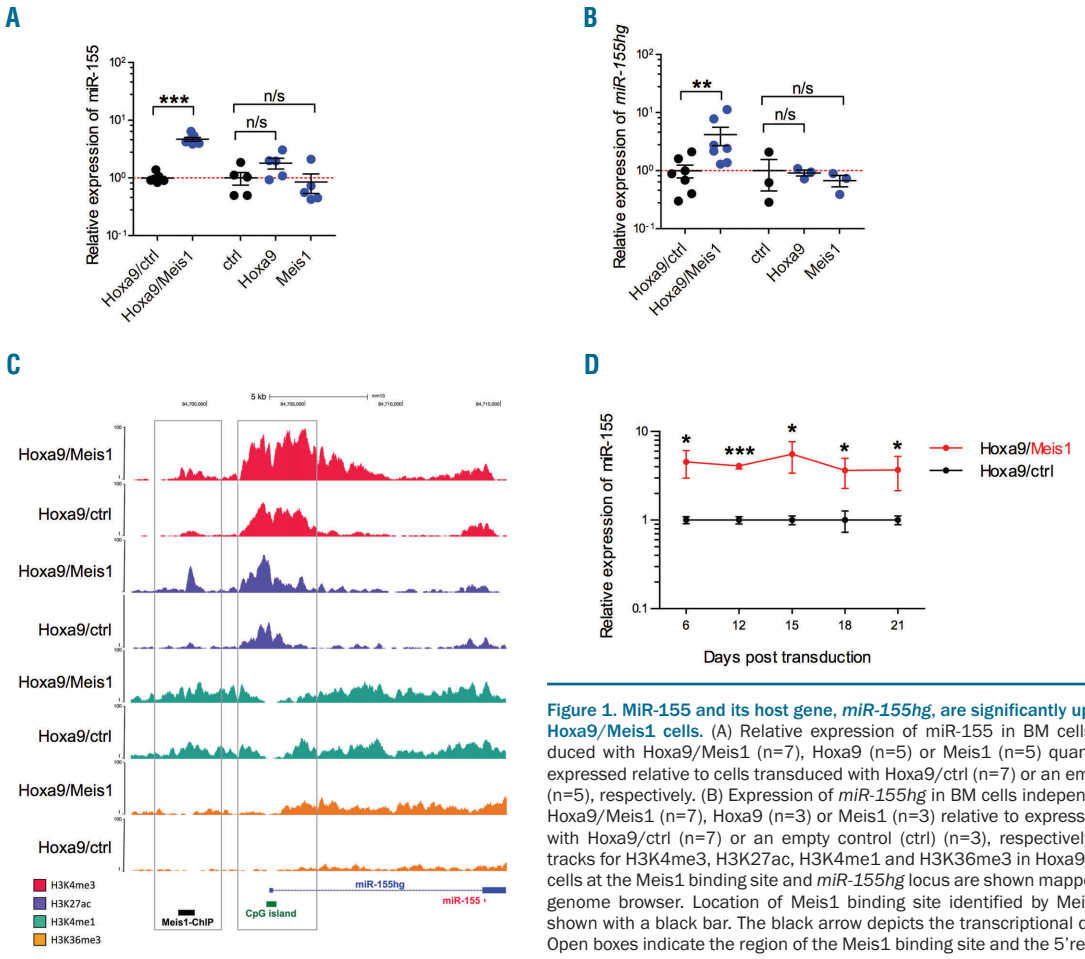


Figure 1. MiR-155 and its host gene, *miR-155hg*, are significantly upregulated in leukemic Hoxa9/Meis1 cells. (A) Relative expression of miR-155 in BM cells independently transduced with Hoxa9/Meis1 (n=7), Hoxa9 (n=5) or Meis1 (n=5) quantified by RT-qPCR and expressed relative to cells transduced with Hoxa9/ctrl (n=7) or an empty control vector (ctrl) (n=5), respectively. (B) Expression of *miR-155hg* in BM cells independently transduced with Hoxa9/Meis1 (n=7), Hoxa9 (n=3) or Meis1 (n=3) relative to expression in cells transduced with Hoxa9/ctrl (n=7) or an empty control (ctrl) (n=3), respectively. (C) ChIP-sequencing tracks for H3K4me3, H3K27ac, H3K4me1 and H3K36me3 in Hoxa9/Meis1 and Hoxa9/ctrl cells at the Meis1 binding site and *miR-155hg* locus are shown mapped to the mouse mm10 genome browser. Location of Meis1 binding site identified by Meis1 ChIP-sequencing is shown with a black bar. The black arrow depicts the transcriptional direction of *miR-155hg*. Open boxes indicate the region of the Meis1 binding site and the 5'region of the *miR-155hg*. (D) The kinetics of miR-155 expression measured by RT-qPCR over time in n=3 biological replicates of Hoxa9/Meis1 transduced cells relative to Hoxa9/ctrl cells. Statistical significance was calculated using the Student's *t*-test (two-tailed). See also *Online Supplementary Tables S5-S7*.

when compared to Hoxa9/ctrl, whereas red blood cells (RBCs) and platelet levels were similar in deceased mice transplanted with Hoxa9/ctrl, Hoxa9/miR-155 and Hoxa9/Meis1 (*Online Supplementary Figure S2B*). Flow cytometry analysis of BM cells from deceased Hoxa9/miR-155 mice showed a lower proportion of c-kit-expressing cells in comparison to BM from Hoxa9/Meis1 mice, but elevated c-kit$^+$ cells compared to Hoxa9/ctrl, verifying the immature blast accumulation in these mice (Figure 2D). These results show that miR-155 partially recapitulates the pro-leukemic effects of Meis1, leading to AML with intermediate latency.

MiR-155 promotes the *Hoxa9* gene expression program

To dissect the molecular differences between the long-latency Hoxa9/ctrl, the intermediate-latency Hoxa9/miR-155 and the short-latency Hoxa9/Meis1 cells, we performed mRNA expression arrays. The fold change of the deregulated genes in Hoxa9/miR-155 and Hoxa9/Meis1 cells was defined relative to the Hoxa9/ctrl cells. In Hoxa9/miR-155 cells 223 genes and in Hoxa9/Meis1 cells 381 genes were differentially expressed when compared to Hoxa9/ctrl cells (*Online Supplementary Table S4*). Analysis of the overlapping genes deregulated in Hoxa9/miR-155 and Hoxa9/Meis1 cells displayed a strikingly similar gene expression pattern with 64 common genes, of which 58 genes followed the same expression direction in both cell lines (Figure 3A and *Online Supplementary Table S4*), further indicating a role for miR-155 in effecting the leukemogenic potential of Hoxa9/Meis1. Of the 223 differentially expressed genes in Hoxa9/miR-155 cells, only 29 were upregulated, and of the 194 downregulated genes, 114 were validated targets of miR-155 (*Online Supplementary Table S5*), based on an analysis using TarBase.[32] Among these downregulated genes were a number of predicted miR-155 targets linked to hematological malignancies, including the previously validated miR-155 targets *Csf1r, Jarid2* and *Picalm*[8] as well as targets associated with myeloid cell differentiation, such as *Anpep* (CD13).[33] Gene ontology and pathway analysis of all differentially expressed genes in Hoxa9/miR-155 cells revealed immune- and cell adhesion-related processes as the main affected pathways (*Online Supplementary Table S6*).

To further define the effects of miR-155 overexpression on Hoxa9-mediated pathways, we compared the differentially expressed genes of the Hoxa9/miR-155 and Hoxa9/Meis1 cells (normalized to Hoxa9/ctrl, as described above) to a previously published Hoxa9-medi-

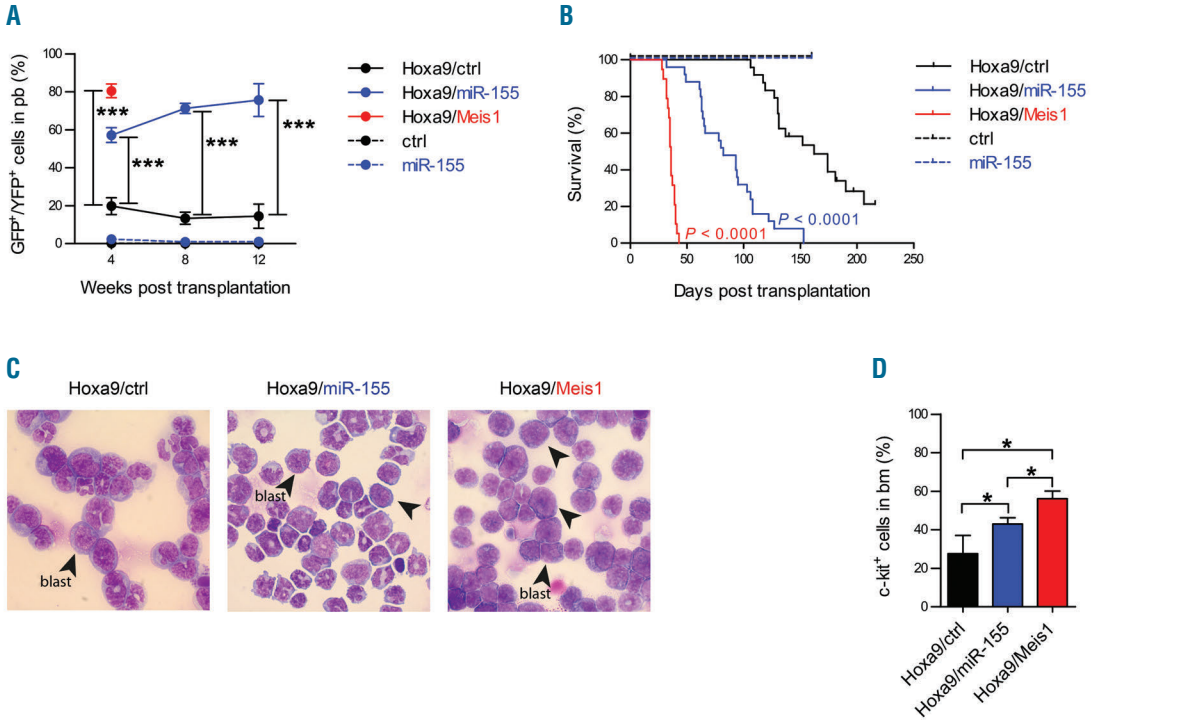


Figure 2. MiR-155 cooperates with Hoxa9 to induce leukemia *in vivo*. (A) Engraftment kinetics in peripheral blood (PB) of mice transplanted in three to four individual experiments with independently generated cell lines for each experiment of Hoxa9/ctrl (week 4 n=25; week 8 n=23; week 12 n=8), Hoxa9/miR-155 (week 4 n=24; week 8 n=20; week 12 n=5), Hoxa9/Meis1 (n=23), miR-155 (n=16) or a control vector (n=16) for each experiment. Engraftment was measured by flow cytometry analysis and data is shown as mean of green fluorescent protein (GFP)$^+$ or yellow fluorescent protein (YFP)$^+$ cells at 4, 8 and 12 weeks after transplantation. (B) Survival curves for cohorts of mice transplanted with independently generated Hoxa9/ctrl (n=25), Hoxa9/miR-155 (n=25) and Hoxa9/Meis1 (n=24) cells *via* a minimum of four individual transplantation experiments and transplantation of cells overexpressing miR-155 (n=16) or control (n=16) from three individual experiments. (C) Representative microscopic photographs of Wright-stained bone marrow (BM) cells (magnification 50x) are shown for mice that succumbed to leukemia. (D) Percentage of c-kit-expressing cells measured *via* flow cytometry in the BM cells of mice that developed leukemia after transplantation of Hoxa9/ctrl (n=8), Hoxa9/miR-155 (n=13) or Hoxa9/Meis1 (n=13) cells. See also *Online Supplementary Figure S1* and *Online Supplementary Figure S2*.

ated gene expression dataset derived from a conditional Hoxa9 cell line by Huang *et al.*[34] There were 152 overlapping genes between Hoxa9/miR-155 and the Hoxa9-mediated gene expression dataset, of which 145 genes showed the same expression direction (i.e., pro Hoxa9-mediated program). Hoxa9/Meis1 cells and the Hoxa9-mediated gene expression dataset shared 219 overlapping genes, the majority of which (191 genes) followed the same expression direction as the Hoxa9-mediated program (Figure 3B and *Online Supplementary Table S5*). To evaluate if miR-155 expression is functionally redundant in the setting of Hoxa9 and Meis1 overexpression, as suggested by the expression analysis, we engineered overexpression of miR-155 in Hoxa9/Meis1 cells (Hoxa9/Meis1/miR-155) that were transplanted into syngeneic recipients. Additional miR-155 overexpression significantly accelerated the formation of AML (Figure 3C), further emphasizing the oncogenic contribution of miR-155 to leukemogenesis. Our data indicate that miR-155 partially activates a similar transcriptional program as Meis1 together with Hoxa9, leading to enhanced leukemogenesis, therefore suggesting that it is one of the key miRNAs within the Hoxa9/Meis1 leukemic program.

MiR-155 expression is not hierarchical in Hoxa9/Meis1 induced AML

We further hypothesized that miR-155 expression reflects a hierarchical structure within Hoxa9/Meis1 leukemias, comparable to its differential expression during normal hematopoiesis (*Online Supplementary Figure S3A*) where its levels decrease with differentiation. Thus, we sorted independently generated Hoxa9/Meis1 cells into subpopulations defined by c-kit, Gr-1 and Mac-1 expression (*Online Supplementary Figure S3B*), with LICs being enriched in the immature c-kit$^+$Gr-1-Mac-1$^-$ population as previously reported by Gibbs *et al.*[35] and validated *in vivo* (*Online Supplementary Figure S3C*). No difference in miR-155 expression was found among the subpopulations (Figure 3D), indicating that miR-155 might not be critical for LICs in the context of Hoxa9/Meis1.

MiR-155 is dispensable for the onset and progression of AML

Albeit miR-155 has become a pharmacological target in hematological malignancies,[13,36-38] *in vivo* data showing the therapeutic benefit of direct depletion of miR-155 from AML cells is lacking. To explore the relevance of miR-155 in AML, we investigated the dependency of Hoxa9/Meis1-induced leukemias on miR-155 expression by overexpressing Hoxa9/Meis1 in BM cells of mice lacking miR-155 expression (Hoxa9/Meis1$^{155-/-}$). Although the proliferation rate of Hoxa9/Meis1$^{155-/-}$ cells showed no difference compared to wt cells transduced with Hoxa9/Meis1 (Hoxa9/Meis1$^{155+/+}$), colony formation capacity was impaired in Hoxa9/Meis1$^{155-/-}$ cells for the first two replates, but was compensated after the third replate and comparable to levels seen for the Hoxa9/Meis1$^{155+/+}$ cells (*Online Supplementary Figure S4A,S4B*). The absence of

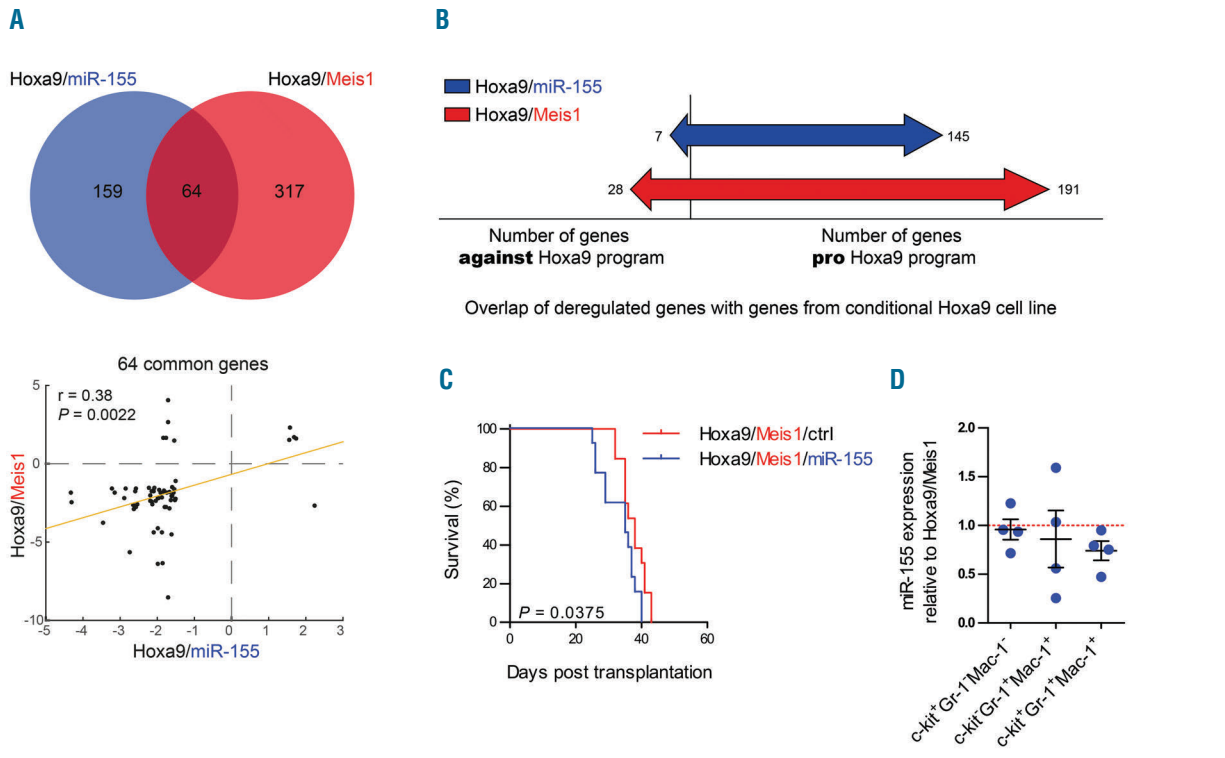


Figure 3. Hoxa9/miR-155 and Hoxa9/Meis1 cells display a similar gene expression pattern. (A) Upper panel shows overlap of differentially expressed genes in Hoxa9/miR-155 and Hoxa9/Meis1 cells. The fold change of differentially expressed genes was calculated relative to Hoxa9/ctrl cells. The scatter plot below shows the fold change of common genes between Hoxa9/Meis1 and Hoxa9/miR-155. The significance and correlation coefficient (r) was calculated using Pearson's correlation coefficient. (B) Overlap between gene expression profile of Hoxa9/miR-155 and Hoxa9/Meis1 cells with the Hoxa9-mediated gene expression dataset from a conditional Hoxa9 cell line (Huang *et al.*).[34] (C) Survival curves of mice transplanted with two biological replicates of Hoxa9/Meis1/ctrl or Hoxa9/Meis1/miR 155 cells (n=13/arm). (D) MiR-155 expression levels in the depicted sorted subpopulations of Hoxa9/Meis1 cells shown relative to expression in Hoxa9/Meis1 bulk cells. For each subpopulation n=4 biological replicates were analyzed, except for the c-kit Gr 1+Mac 1+ subpopulation, where only four replicates were available. See also *Online Supplementary Figure S3*, *Online Supplementary Table S4* and *Online Supplementary Table S5*.

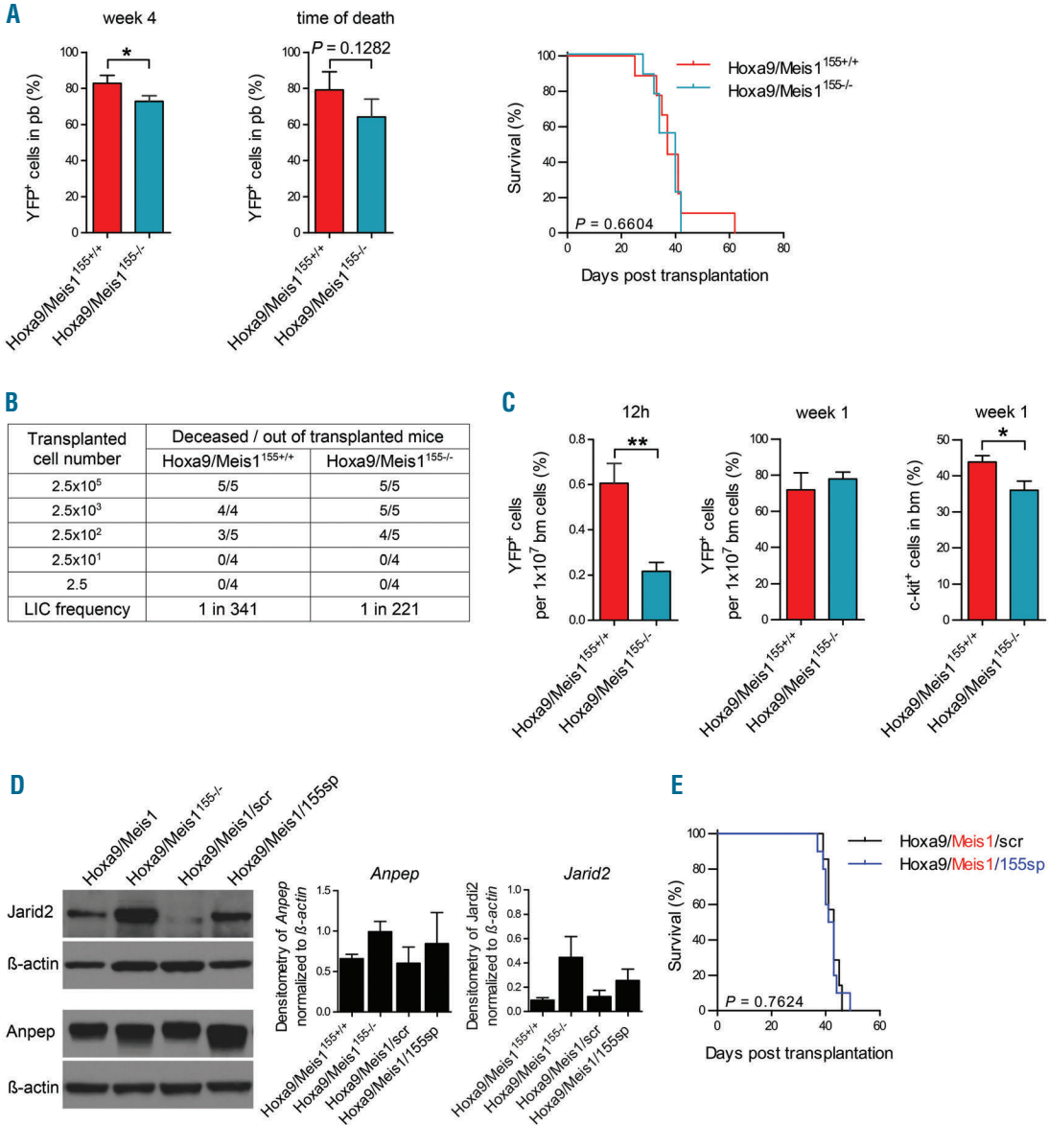
miR-155 led to lower engraftment levels in mice transplanted with Hoxa9/Meis1$^{155-/-}$ after four weeks, but at the time of death these levels reached that of the Hoxa9/Meis1$^{155+/+}$ cells, mirroring the results from the colony-forming assay and resulting in similar survival kinetics as those for Hoxa9/Meis1$^{155+/+}$ (Figure 4A). MiR-155 has recently been linked to a leukemic stem cell signature.[39] Therefore, to exclude differences in LIC frequency between Hoxa9/Meis1$^{155-/-}$ and Hoxa9/Meis1$^{155+/+}$, we performed limiting dilution transplantations where no significant difference was detected (Figure 4B). In order to explore the possibility of impaired early leukemic cell homing, we sacrificed Hoxa9/Meis1$^{155-/-}$ and Hoxa9/Meis1$^{155+/+}$ transplanted mice 12 hours post-transplantation. Hoxa9/Meis1$^{155-/-}$ cells showed significantly less yellow fluorescent protein (YFP)$^{+}$ cells, indicating a role of miR-155 in homing of AML cells (Figure 4C, left panel). This observation was already reversed after one week, with similar YFP levels, despite lower c-kit levels in Hoxa9/Meis1$^{155-/-}$ cells (Figure 4C, middle and right panel), indicating differences in LIC pathophysiology. Molecular differences between Hoxa9/Meis1$^{155-/-}$ and Hoxa9/Meis1$^{155+/+}$ cells were explored by mRNA expression arrays. Since miRNAs act as rheostats and induce small gene expression differences, we investigated genes with a fold change >1.2 and *P*-value cutoff of 0.05. In Hoxa9/Meis1$^{155-/-}$ cells 295 genes were downregulated and 115 upregulated compared to Hoxa9/Meis1$^{155+/+}$ (*Online Supplementary Table S7*). As expected, miR-155 targets *Jarid2* and *Anpep*, which are involved in the regulation of hematopoietic cell differentiation and were downregulated in Hoxa9/miR-155 cells, were upregulated in Hoxa9/Meis1$^{155-/-}$ cells (*Online Supplementary Table S7*). To further investigate if wt Hoxa9/Meis1 cells are addicted to miR-155, we combined an immortalized Hoxa9/Meis1 cell line with a miR-155 sponge vector[26] (Hoxa9/Meis1/155sp) or a scrambled vector (Hoxa9/Meis1/scr) as control. MiR-155 levels were significantly reduced by the miR-155 sponge (*Online Supplementary Figure S4C*). Reduced protein levels of the miR-155 targets, *Jarid2* and *Anpep*,[8] which we previously identified by mRNA expression arrays (*Online Supplementary Table S4* and *Online Supplementary Table S7*), were confirmed with immunoblotting in Hoxa9/Meis1/155sp sponge as well as Hoxa9/Meis1$^{155-/-}$ cells when compared to wt Hoxa9/Meis1 cells (Figure 4D). However, no difference in survival was observed between Hoxa9/Meis1/scr and Hoxa9/Meis1/155sp transplanted mice (Figure 4E). Taken together, these results indicate that the oncogenic program of Meis1 is not dependent on miR-155, as shown in the knockout experiments, and can overcome a 50% reduction of miR-155 expression.

Lower expression of miR-155 in AML patients compared to HSPCs

Based on our data in the Hoxa9/Meis1 model, we sought to corroborate our findings in human AML samples. Within The Cancer Genome Atlas Acute Myeloid

Leukemia (TCGA-LAML) dataset,[1] miR-155 expression levels were significantly increased in all AML patients when compared to the global mean of all miRNAs, and particularly in AML patients with mutated FLT3 (Figure 5A), reinforcing the fact that miR-155 is especially abundant in CN-AML. We further analyzed the correlation between HOXA9 and MEIS1 with miR-155 levels within the TCGA-LAML dataset. Supporting our hypothesis, CN-AML with mutated NPM1, known to have high HOXA9 and MEIS1 levels,[40] displayed a significant correlation between *HOXA9, MEIS1* and miR-155 levels (*Online Supplementary Figure S5A,S5B*) within the TCGA-LAML dataset.

To investigate the relationship of our findings in human AML to normal hematopoiesis, we quantified miR-155 levels by RT-qPCR in diagnostic samples from CN-AML and AML with t(11q23) in relation to human CD34$^+$ cord blood cells, total BM and granulocyte samples from



Transplanted cell number	Deceased / out of transplanted mice	
	Hoxa9/Meis1$^{155+/+}$	Hoxa9/Meis1$^{155-/-}$
2.5x10^5	5/5	5/5
2.5x10^3	4/4	5/5
2.5x10^2	3/5	4/5
2.5x10^1	0/4	0/4
2.5	0/4	0/4
LIC frequency	1 in 341	1 in 221

Figure 4. Absence or depletion of miR-155 does not alter leukemogenicity of Hoxa9/Meis1 cells. (A) Engraftment in peripheral blood (PB) after 4 weeks (left panel), at the time of death (middle panel, n=7/arm) and survival (right panel) of mice transplanted in two independent experiments with two biological replicates of Hoxa9/Meis1$^{155+/+}$ (n=9) or Hoxa9/Meis1$^{155-/-}$ (n=9) cells. Percentage of yellow fluorescent protein (YFP)$^+$ cells was measured by flow cytometry in PB of mice. (B) Limiting dilution assay of Hoxa9/Meis1$^{155+/+}$ and Hoxa9/Meis1$^{155-/-}$ transplanted cells with indicated numbers of mice transplanted for each arm. Leukemia initiating cell frequency was calculated using L-cal™. (C) Left: homing percentage of Hoxa9/Meis1$^{155+/+}$ and Hoxa9/Meis1$^{155-/-}$ (n=5/arm) cells transplanted into non-irradiated recipient mice and assessed 12h post transplantation. Middle: percentage of Hoxa9/Meis1$^{155+/+}$ and Hoxa9/Meis1$^{155-/-}$ (n=5/arm) cells transplanted into non-irradiated recipient mice and assessed one week post transplantation. Right: percentage of c-kit positive cells in Hoxa9/Meis1$^{155+/+}$ and Hoxa9/Meis1$^{155-/-}$ (n=5/arm) cells transplanted into non-irradiated recipient mice and assessed one week post transplantation. (D) Left: western blot of Jarid2 and Anpep in Hoxa9/Meis1$^{155+/+}$ and Hoxa9/Meis1$^{155-/-}$ as well as Hoxa9/Meis1/155 sponge and Hoxa9/Meis1/scr-transduced cells. Right: densitometry of Anpep and Jarid2 normalized to β-actin (gene/β-actin) using the ImageJ software (n=2 biological replicates). (E) Survival curves of two independent transplantation experiments, where 1000 cells of Hoxa9/Meis1/155 sponge (n=10) or Hoxa9/Meis1/scr, (n=7) cells were transplanted. See also *Online Supplementary Figure S4* and *Online Supplementary Table S7*. BM: bone marrow; LIC: Leukemia-initiating cell.

healthy donors. Interestingly, the expression of miR-155 was significantly higher in CD34+ cells compared to the profiled AML subgroups (Figure 5B). We further investigated whether miR-155 is differentially expressed within AML subpopulations based on their differentiation status, reinforcing its potential as a therapeutic target in AML. For this purpose, we sorted subpopulations from seven randomly chosen AML patients according to their maturation states. In line with our findings in the Hoxa9/Meis1 cell lines, there were no differences in miR-155 expression among the subpopulations (Figure 5C). We confirmed this finding in CN-AML patient samples (n=17) (*Online Supplementary Figure S5C*), reinforcing the concept that miR-155 enhances, rather than drives, leukemogenesis.

Discussion

Screening a Hoxa9 and Hoxa9/Meis1-based AML progression model, we found miR-155 to be the most significantly upregulated miRNA in leukemic Hoxa9/Meis1 cells. MiR-155 is a known oncogene that accelerates the formation of lymphomas and expansion of myeloid cells when overexpressed.[8,29]

Considering the relevance of the HOXA9/MEIS1 axis in AML, the full extent of pro-leukemic targets of HOXA9 and MEIS1 is not yet fully understood, which leaves this axis undruggable. Several of these targets, such as Sytl1[41] and Syk,[42] support leukemic cell homing and engraftment and promote leukemogenesis. Thus far, few miRNAs, including miR-196b,[43] miR-21[44] and miR-146a[42] have been linked with Hoxa9 and Meis1 regulation. Huang *et al.* identified a Meis1 binding site nearly 4kb upstream of the transcriptional start site of *mir155hg* (Bic), the host gene of miR-155.[34] The data herein confirms, for the first time, the direct binding of Meis1 to this region that displays the hallmarks of an activated enhancer in Hoxa9/Meis1 cells. To our knowledge, miR-155 is the first discovered miRNA

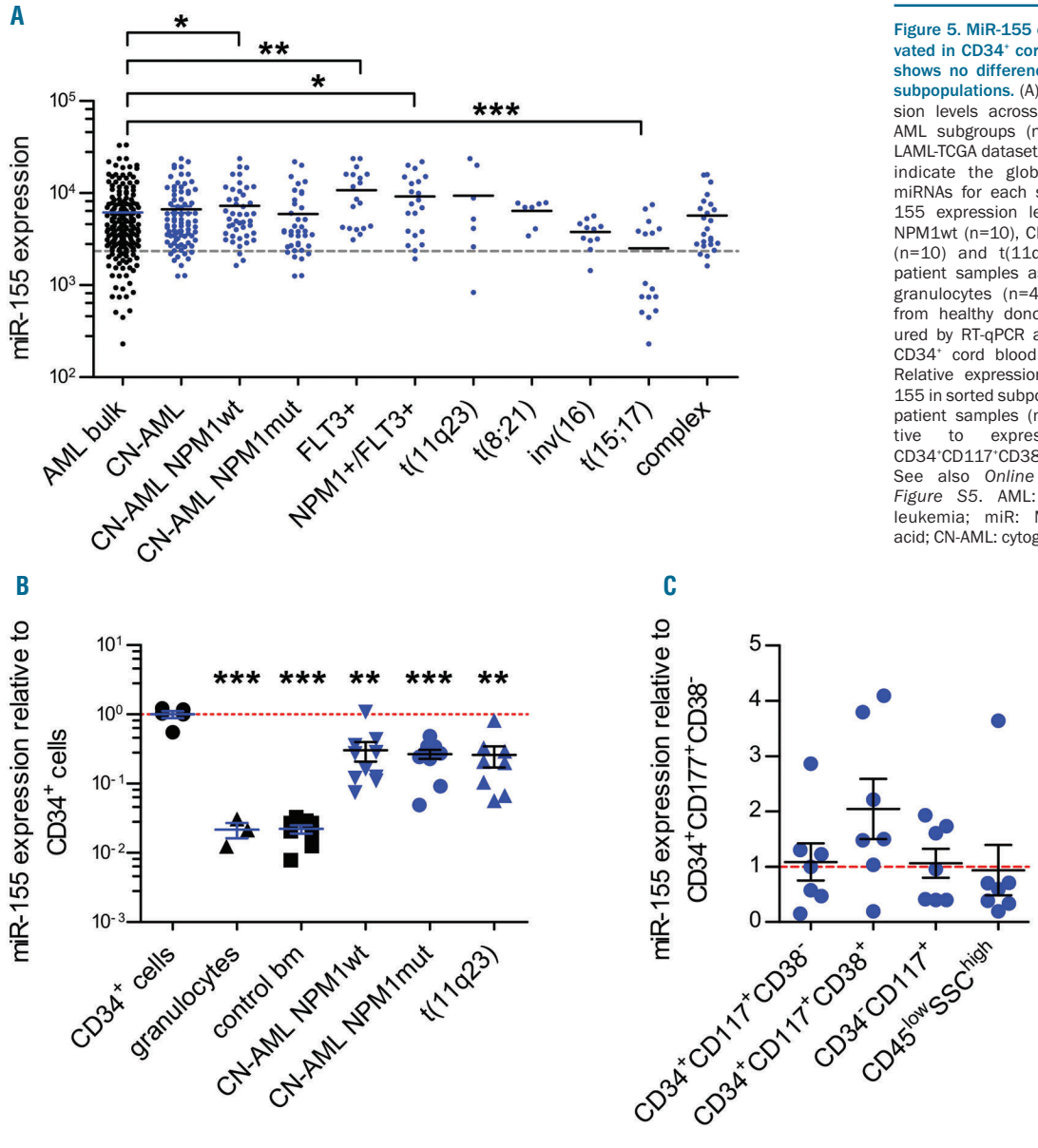


Figure 5. MiR-155 expression is elevated in CD34+ cord blood cells but shows no difference between AML subpopulations. (A) MiR-155 expression levels across various genetic AML subgroups (n=187) from the LAML-TCGA dataset. The gray dashes indicate the global mean of all miRNAs for each sample. (B) MiR-155 expression levels in CN-AML NPM1wt (n=10), CN-AML NPM1mut (n=10) and t(11q23) (n=8) AML patient samples as well as sorted granulocytes (n=4) and total BM from healthy donors (n=8), measured by RT-qPCR and compared to CD34+ cord blood cells (n=5). (C) Relative expression levels of miR-155 in sorted subpopulations of AML patient samples (n=7) shown relative to expression in the CD34+CD117+CD38- subpopulation. See also *Online Supplementary Figure S5*. AML: acute myeloid leukemia; miR: Micro-ribonucleic acid; CN-AML: cytogenetically normal

which is a direct downstream target of Meis1, highlighting that the oncogenic potential of Meis1 is based on coding and non-coding genes.

Our *in vitro* and *in vivo* studies demonstrate the collaborative and oncogenic potential of miR-155. However, the combination of Hoxa9 and miR-155 overexpression only partially recapitulated the leukemic potential of Hoxa9 and Meis1 overexpression, implying that additional genetic factors are needed for the generation of aggressive AML. For example, multiple profiling approaches have previously associated miR-155 with mutated *FLT3 (FLT3-ITD)* in AML.[3,13,45,46] MiR-155 has been attributed to oncogenic or tumor suppressor functions in AML. For example, Palma *et al*. highlighted a tumor suppressor role for miR-155 through the induction of apoptosis and myeloid differentiation in the AML cell line OCI-AML[3,47] whereas several groups have suggested the inhibition of miR-155 as a therapeutic approach in AML.[37,46,48-50] It seems that the function of miR-155 could be context-dependent. Narayan *et al*. recently showed a dose-dependent role for miR-155 in AML, which might partially explain the published discrepancies.[30] In the context of Hoxa9/Meis1-induced leukemia, a very potent AML model, we did not detect clonal selection based on endogenous miR-155 levels through enforced expression.[30] This might be attributed to: 1) a very early selection of miR-155 clones with intermediate expression levels, 2) and/or to relatively constant ectopic expression levels, and 3) the specific (model-dependent) Hoxa9/Meis1 cell context in which the absence of miR-155 did not impact leukemogenesis.

While miR-155 is overexpressed in a range of hematological malignancies, it is still unclear how miR-155 promotes leukemogenesis. We show that overexpression of miR-155 enhances the leukemic properties of Hoxa9 through downregulation of its targets, the majority of which are known tumor suppressor genes associated with processes such as myeloid differentiation, for example *Jarid2* and *Klf4*.[8,51,52] The absence or partial removal of miR-155 did not impede Hoxa9 and Meis1-induced AML development, however, since we did not deplete miR-155 in other human AML models, a critical role for this miRNA in leukemogenesis cannot be fully excluded. Of note, although miR-155 is highly expressed in murine HSPCs, miR-155$^{-/-}$ mice did not show impaired myeloid differentiation or any perturbations in the HSPC compartment.[11] Herein, we linked the absence of miR-155 to impaired early homing of Hoxa9/Meis1 cells, a previously unrecognized function of miR-155, which was reversed after only one week post transplantation, but which supports our recent findings demonstrating the necessity of miR-155 for the mobilization of HSPCs.[14] However, the impact of miR-155 on homing in the context of human AML development is of yet undetermined.

In order to translate our findings to AML patients, we further determined miR-155 levels in AML subtypes with elevated *HOXA9* and *MEIS1* transcript levels in addition to healthy donor BM cells. CD34^{+} cells exhibited significantly higher miR-155 levels compared to AML patients, suggesting that the profiled AML blasts were more differentiated than human HSPCs. Albeit high miR-155 expression levels were associated with an inferior overall survival in CN-AML samples[49] and being upregulated in FLT3-ITD positive AML[3,45,47] or French-American-British (FAB) M4/5 AML,[53] we did not detect significantly altered miR-155 levels in sorted AML subpopulations. The fact that miR-155 is directly regulated by Hoxa9/Meis1 and accelerates Hoxa9/Meis1 AML, but is nevertheless dispensable for the initiation and progression of leukemia, suggests that miR-155 accelerates transformation rather than acting as a driver of leukemogenesis, supporting our previous findings in MLL-rearranged AML where the absence of miR-155 did not impact AML formation and progression.[15] However, the function of miR-155 in AML still remains controversial, as Wallace *et al*. recently showed that miR-155 is relevant for human MV4-11 cell line growth[38] as well as colony formation of AML samples with mutated FLT3 *in vitro*.[13] Of note, inhibition of FLT3 signaling with SU14813 did not alter miR-155 expression in the same MV4-11 AML cell line,[45] suggesting that the transcriptional activation of miR-155 may be independent of FLT3 signaling. MiR-155 has recently been included in a predictive 4-miRNA signature,[39] which is in line with its leukemia-enhancing role, as shown in the context of Hoxa9 and Hoxa9/Meis1 *in vivo*. However, its potential as an *in vivo* driver has not yet been validated.

Taken together, our results show that miR-155 accelerates, but is not required for the induction of AML and that its absence leads to impaired engraftment/homing of AML cells without affecting the onset of AML.

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