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ORIGINAL ARTICLE

miR-9 is a tumor suppressor in pediatric AML with t(8;21)

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MicroRNAs (miRNAs) play a pivotal role in the regulation of hematopoiesis and development of leukemia. Great interest emerged in modulating miRNA expression for therapeutic purposes. In order to identify miRNAs, which specifically suppress leukemic growth of acute myeloid leukemia (AML) with t(8;21), inv(16) or mixed lineage leukemia (*MLL*) rearrangement by inducing differentiation, we conducted a miRNA expression profiling in a cohort of 90 cytogenetically characterized, *de novo* pediatric AML cases. Four miRNAs, specifically downregulated in *MLL*-rearranged, t(8;21) or inv(16) AMLs, were characterized by their tumor-suppressive properties in cell lines representing those respective cytogenetic groups. Among those, forced expression of miR-9 reduced leukemic growth and induced monocytic differentiation of t(8;21) AML cell lines *in vitro* and *in vivo*. The tumor-suppressive functions of miR-9 were specifically restricted to AML cell lines and primary leukemic blasts with t(8;21). On the other hand, these functions were not evident in AML blasts from patients with *MLL* rearrangements. We showed that miR-9 exerts its effects through the cooperation with let-7 to repress the oncogenic *LIN28B*/*HMGA2* axis. Thus, miR-9 is a tumor suppressor-miR which acts in a stringent cell context-dependent manner.

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INTRODUCTION

Pediatric acute myeloid leukemia (AML) is a heterogeneous disease that is morphologically and cytogenetically classified into different subtypes. At present, cytogenetic aberrations along with treatment response are the most important prognostic factors.[1] In AML, well-known chromosomal aberrations that predict the differences in survival include t(15;17)(q22;q21) (*PML-RARα*), t(8;21)(q22;q22) (*RUNX1-RUNX1T1*), inv(16)(p13q22) (*CBF-MYH11*), t(7;12) and mixed lineage leukemia (*MLL*) rearrangements.[1,2] In addition, molecular changes that cannot be detected by classical karyotyping, such as *WT1*, *NPM1*, *CEBPA* and *DNMT3A* mutations, have an influence on the biology and prognosis in pediatric as well as adult AML.[1,3] Recently, alterations in the expression program caused by transcriptional or post-transcriptional mechanisms have been demonstrated to be responsible for leukemogenesis.[4] Altered transcription can be caused by misdirected epigenetic modifications, including histone methylation and DNA methylation, or dysregulated microRNA (miRNA) expression, which perturbs post-transcriptional gene regulation by interference with mRNAs.[5] MiRNA expression profiles or signatures have been identified in solid tumors as well as hematopoietic malignancies, including adult AML.[6,7] Some miRNAs, such as miR-9, miR-181 and miR-212 have been shown to be able to predict outcome in adult AML.[8–10] Subsequent functional studies revealed their central regulatory role in hematopoietic differentiation and their contribution to leukemic transformation.[4] For instance, miR-196b is aberrantly overexpressed in *MLL*-rearranged AML,[11] and its knockdown by antagomirs overturned *MLL*-fusion-mediated cell immortalization.[12] Similarly, miR-29b and miR-125b were shown to contribute to myeloid leukemogenesis.[13–15] In contrast, higher miR-181a expression is associated with favorable outcome especially in adult AML with normal karyotype,[10] while ectopic expression of miR-181a sensitizes AML cell lines to chemotherapy *in vivo* and *in vitro*.[16] These insights open new avenues for therapeutic intervention: modulation of a deregulated miRNA, which specifically induces differentiation of leukemic blasts while sparing normal hematopoiesis, could be exploited therapeutically. This would mimic the effect of ATRA (all-trans-retinoic acid) treatment in acute promyelocytic leukemia expressing the PML-RARα fusion protein.

However, for pediatric AML, reports on miRNA expression signatures are scarce. Subtle differences in miRNA expression patterns between pediatric and adult samples suggests the importance of performing a large-scale miRNA expression screening in pediatric AML patients.[11]

Here we describe a large-scale miRNA expression profiling in a representative cohort of well-characterized pediatric AML patients in order to identify specific, clinically relevant, tumor-suppressive miRNAs. This study discovered that miR-9 suppresses leukemic growth of t(8;21) AML *in vitro* and *in vivo*, while inducing monocytic differentiation by interfering with the *LIN28B*/let-7/*HMGA2* axis.

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MATERIALS AND METHODS

Patient samples and cell lines

Viably frozen diagnostic bone marrow or peripheral blood samples from a selection of 90 *de novo* pediatric AML cases were provided by the Dutch Childhood Oncology Group (DCOG), the AML 'Berlin-Frankfurt-Münster' Study Group (AML-BFM-SG), the Czech Pediatric Hematology Group (CPH) and the St. Louis Hospital in Paris, France. Samples were chosen to represent the most common and relevant cytogenetic groups, that is, t(8;21), inv(16), t(7;12), t(15;17) as well as the *MLL*-rearranged pediatric AML and were selected based on the availability of high-quality RNA. Normal karyoype and 'other' cytogenetic subtypes of AML were not included in the current analyses based on the gross molecular heterogeneity. Each study group performed central morphological reviews according to the FAB classification. Ficoll-separated bone marrow mononuclear cells were collected from four children without malignant tumors or bone marrow abnormalities (Sophia Children's Hospital, Rotterdam). $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) were obtained from cord blood of healthy donors and isolated using CD34-immunomagnetic microbeads (Miltenyi, Bergisch Gladbach, Germany). Informed consent was obtained from all patients, after Institutional Review Board approval, according to national law and regulations and in accordance to the Declaration of Helsinki. Samples were enriched to contain at least 80% leukemic cells as previously described.[17]

Cell lines NB4, THP-1, ME-1, SKNO-1 and KASUMI-1 were obtained from the German National Resource Center for Biological Material (DSMZ, Braunschweig, Germany) and cultured as recommended. Differentiation of KASUMI-1, SKNO-1 and THP-1 cells was induced by 50 nM Calcitriol (Sigma-Aldrich, St Louis, MO, USA) and 10 ng/ml GM-CSF (Preprotech, Rocky Hill, NJ, USA) over 7 days. Differentiation of NB4 cells was induced by 1 mM ATRA (Sigma-Aldrich) and 10 ng/ml G-CSF (Preprotech).

Xenograft mouse model

NOD.Cg-Rag1t^{m1Mom} Il2rg$^{tm1Wjl/SzJ}$ (NRG) and Foxn1$^{nu/J}$ (nude) mice (Jackson Laboratory, Bar Harbor, ME, USA) were maintained in a pathogen free environment. All experimental procedures using these mice were performed in accordance with protocols approved by the local authorities (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit). For competitive *in vivo* assay, 1×10^7 KASUMI-1 cells (ratio 2:1 of transduced vs untransduced cells) were injected i.f. per animal into a cohort of 10 recipients. Recipients were randomly assigned. After 28 days, mice were sacrificed. Leukemic cells were isolated from femurs and analyzed as described.[18] In another setting, 1×10^7 transduced KASUMI-1 cells were injected s.c. into Foxn1$^{nu/J}$ mice, together with Matrigel (BD, Franklin Lakes, NJ, USA). Tumor mass was evaluated after 6 weeks.

MiRNA and mRNA expression profiling and RT-qPCR

Total cellular RNA was isolated using Trizol reagent (Invitrogen, Breda, The Netherlands) as previously described.[19] MiRNA expression was measured on Taqman Array MicroRNA Cards v2.0 using Taqman technology in the 7900 HT Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' protocol. mRNA expression profiling was performed using Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's guidelines. For more details see supplementary Methods. All microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) with GEO Series accession numbers GSE52541.

Lentiviral transduction

MiRNAs were cloned into a modified Lentiviral Gene Ontology (LeGO) as previously described.[20] As control a non-silencing shRNA inside the miR-30 backbone (ThermoFisher Scientific Inc., Waltham, MA, USA) was used. Stable miRNA knockdown was achieved using the sponge technology.[21] In brief, 12 specific target sites of miR-9 as an artificial 3'UTR were inserted into the lentiviral pLBid-GIP/C vector[22] downstream of the SFFV-driven mCherry cassette, while an antisense CMV-driven GFP-IRES-Puro served as selection marker (Supplementary Figure 4b). The non-silencing sponge (SP-sc) control sequence was adapted from Loya *et al*.[21] THP-1 cells with higher endogenous miR-9 levels (Supplementary Figure 3) were used to monitor functionality of the SP-9 decoy by decreased mCherry fluorescence (Supplementary Figure 4c). The following shRNAs in the pLKO.1 (ThermoFisher) were used for target validation: *HMGA2*#1 (TRCN0000021964), *HMGA2*#2 (TRCN00000021966), *HMGA2*#3 (TRCN0000021967) and *LIN28B*#1 (TRCN0000122599), *LIN28B*#2 (TRCN0000143619) and *LIN28B*#3 (TRCN0000219859) (Sigma-Aldrich). Transduction and culture of HSPCs and cell lines was performed using lentiviral supernatant, prepared by calcium-phosphate transfection of HEK293T cells as previously described.[14,20] HSPCs were cultured in RPMI (10% FCS and 1% penicillin streptomycin); FLT3L, IL3, IL6, GM-CSF, M-CSF (all Preprotech) for monocytic *in vitro* differentiation, or in the presence of SCF, IL3, GM-CSF, and G-CSF for granulocytic *in vitro* differentiation. For *in vitro* studies, leukemic blasts were maintained, transduced or used for colony-forming assays (HSC003, R&D Systems, Wiesbaden-Nordenstadt, Germany or Collagen-Cult, SCT, Vancouver, Canada) as described.[18,23]

Flow cytometry and cell sorting

Immunophenotyping of differentiated cells was performed by flow cytometry on a Navios 10/3 device (Beckman Coulter, Krefeld, Germany) as previously described[14] using the following antibodies: anti-CD14-PC5.5, anti-CD13-PC5.5, anti-CD11b-PC7, anti-CD14-APC and anti-CD15-PacB (Beckman Coulter), and anti-CD11b-APC (BD Pharmingen, Heidelberg, Germany). Kaluza Software by Beckman Coulter was used for analyses. Cells were sorted on a MoFlo device (Beckman Coulter) as previously described.[14,23]

Apoptosis, proliferation and colony forming assays.

Apoptosis analyses using the Annexin V Apoptosis Detection Kit II were performed according to manufacturer's instructions (BD Pharmingen) and as previously described.[23] Cell viability assays were performed using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) on a Glo Max luminometer (Promega) according to the manufacturer's instructions. For growth competition, transduced GFP^+ and $mCherry^+$ cells were mixed in 1:1 ratio. The fraction of GFP^+ and $mCherry^+$ cells was determined every third day by flow cytometry. Colony-forming assays with transduced cell lines and HSPCs were performed and analyzed as described previously (HSC001 for cell lines, HSC003 for HSPCs, R&D Systems).[20]

Western blot

Cell lysates were prepared and western blot analyses and immunoprecipitation were performed as described previously.[14] The following antibodies were used: LIN28 (clone 13D4.1), HMGA2 (DR1073), (Millipore, EMD Millipore Corporation, Billerica, MA, USA), β-ACTIN (C4), anti-mouse IgG HRP, and anti-rabbit IgG HRP (all Santa Cruz, Dallas, TX, USA).

Table 1. Patient characteristics

	Total n = *90*	
Sex	**N**	**(%)**
Male	46	(51)
Female	44	(49)
FAB		
M0	3	(3)
M1	3	(3)
M2	20	(22)
M3	13	(14)
M4	27	(30)
M5	23	(26)
M6	0	(0)
M7	1	(1)
Cytogenetics		
MLL	35	(39)
t(8;21)	21	(23)
inv(16)	17	(19)
t(15;17)	12	(13)
t(7;12)	5	(6)
	Median	**Range**
Age (years)	9.9	(0.1–17.3)
WBC ($\times 10^9$/L)	35.1	(0–475)

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Luciferase reporter assay

For reporter assays, the 3′ UTR or intron fragments of the respective mRNAs containing the miR-9 binding site were cloned from cDNA into pMIR-REPORT (Ambion, Paisley, UK). 293T cells (2×10^4) were plated and co-transfected with 6.25 ng of the firefly luciferase report vector, 0.25 ng of the control vector containing renilla luciferase pGL4.7 (Promega) and 50 ng miRNA expression vector in 96-well plates by using FuGENE HD (Roche, Basel, Switzerland), according to the manufacturer's protocol. Firefly and

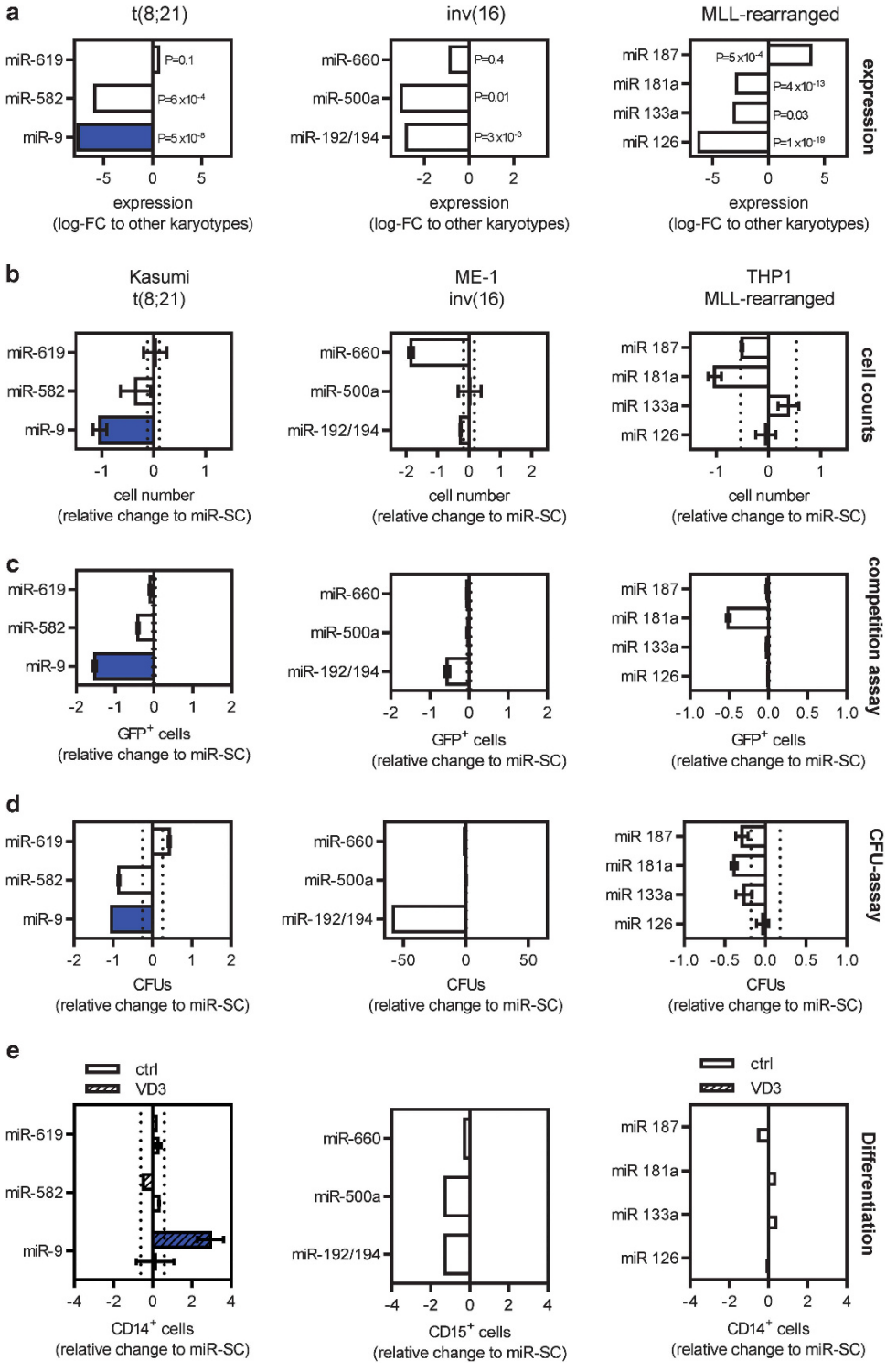


Figure 1. Functional screening of seven deregulated miRNAs or miRNA clusters in three AML cell lines. (**a**) Expression of indicated miRNAs in respective karyotype groups as $\log_2$ fold change over all other karyotypes as measured by TaqMan low-density array (TLDA). (**b**–**e**) All readouts are shown as fold change relative to miR-SC where -1 means one fold reduction (50%), 1 means one fold increase (200%). (**b**) Number of viable, miRNA- or miR-SC-transduced KASUMI-1, ME-1 and THP-1 cells after 3 days in culture determined by luminescence cell viability assay. (**c**) Fraction of GFP^+ miRNA-transduced cells after 15 days of culture in competition with $Cherry^+$ miR-SC-transduced control cells. (**d**) Number of CFUs in methylcellulose-based colony-forming assays of miRNA-transduced cells. (**e**) Percentage of $CD14^+$ or $CD15^+$ miRNA transduced cells, respectively, with or without induction of differentiation by calcitriol (VD3; KASUMI-1, THP-1) after 7 days of culture.

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renilla luciferase activities were measured consecutively by using dual-luciferase assays (Promega), 48 h after the transfection. All experiments were performed in replicates.

Statistical analyses

MiRNA ($n = 253$) DCT values (zero detection values were set at -25) of AML samples ($n = 90$) were clustered using correlation (method spearman) hierarchical clustering (method ward) using the hclust function of R (www.R-project.org) and visualized using heatmap.2.[24] Most discriminative miRNAs were uncovered by detecting significantly differently expressed miRNAs (p.FDR < 0.05) using linear modeling and Baysian P-value estimation based on t-type statistics using the R package limma.[25] For this purpose, each of five cytogenetically defined subgroups were contrasted against the four others.

MiR-9 target genes were indicated by miRecords[26] and considered for further validation if they were predicted by three target prediction algorithms: miRTarget2,[27] PITA[28] and TargetScan.[29] Statistical analyses of experiments were performed using the two-tailed Student's *t*-test for two groups and ANOVA for three or more groups. Analyses were performed with SPSS Statistics Version 19.0 (SPSS Inc., Armonk, NY, USA) or GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). All used tests were two-tailed, and a *P*-value of < 0.05 was considered significant.

RESULTS

In vitro characterization of differentially expressed miRNAs reveals biological significance

AML with t(8;21), inv(16) or *MLL*-rearrangement is characterized by biologically distinct fusion proteins. Together these subgroups represent 44% of the cases.[1] In order to identify miRNAs, which specifically suppress leukemic growth of AML with t(8;21), inv(16) or *MLL*-rearrangement by inducing differentiation, we conducted a miRNA expression profiling in a cohort of 90 cytogenetically characterized, *de novo* pediatric AML cases (Table 1, Supplementary Figure 1-2 and Supplementary Table 1) followed by gain-of-function studies. The decision to further investigate five miRNAs and two miRNA clusters *in vitro* using three cell lines was based on their ranking in the expression screening (specifically downregulated in one karyotype vs all others; Figure 1a and Supplementary Table 1) and on literature review (no reported tumor-suppressive function in AML). The miRNA candidates were cloned in a lentiviral vector system. Ectopic expression of each miRNA was tested in the cell line harboring the respective cytogenetic aberration of the subgroup, where it was downregulated, that is, KASUMI-1 for t(8;21), ME-1 for inv(16) and THP1 for *MLL*-rearranged AML (Figure 1a). To judge the effect, four readouts were used: proliferation was assessed by cell viability assays after 72 h (Figure 1b), cell competition by relative fluorescence on day 15 (Figure 1c), colony-forming capacity by colony-forming unit (CFU) assays (Figure 1d) and differentiation by flow cytometry with and without induction (Figure 1e). For each karyotype, we included one miRNA as a control, which was either insignificantly changed (miR-619 in t[8;21] KASUMI-1 and miR-660 in inv[16] ME-1) or upregulated (miR-187 in *MLL*-rearranged THP1; Figure 1a).

Using this approach, we identified two miRNAs and two miRNA clusters that reduced cell growth and colony-forming capacity upon ectopic expression (Figure 1b–d): two out of two miRNAs in KASUMI-1 (miR-9, miR-582), one out of two in ME-1 (miR-192/194 bicistron) and one out of three in THP1 (miR-181a/b bicistron). Recently, miR-181a was also defined by others[30] to be tumor-suppressive in *MLL*-rearranged leukemia, showing the validity of our results. None of the miRNAs enhanced cell growth and colony-forming capacity upon their ectopic expression.

In KASUMI-1 cells, we identified one miRNA, miR-9, which not only reduced cell growth and colony-forming capacity, but also strongly induced monocytic differentiation in concert with calcitriol (that is, vitamin D3 (VD3); Figure 1e). This suggested the further exploration of the function of miR-9 in t(8;21) AML.

Ectopic expression of miR-9 in KASUMI-1 suppresses growth, colony-forming capacity and synergizes with calcitriol in monocytic differentiation

MiR-9 was repressed in AML patients with t(8;21), while it was expressed to a higher extent in patients with *MLL*-rearrangements (Figure 2a and Supplementary Figure 3a). During normal hematopoiesis miR-9 is only expressed in mature macrophages, and not in stem and/or progenitor cells nor in cells of other mature blood lineages (Figure 2b, from Petriv *et al*.[31]). Those facts, together with the growth arrest and monocytic differentiation upon ectopic miR-9 expression in t(8;21)-positive cell line KASUMI-1, point towards a regulatory function of miR-9 during monopoiesis and an advantage of miR-9-downregulation during t(8;21)-mediated leukemogenesis.

This was further investigated in separate experiments confirming the primary characterization. Consistently, upon ectopic miR-9 expression (137-fold elevation compared with the miR-SC (non-silencing) control, Supplementary Figure 3b) we observed a reduction in growth of KASUMI-1 cells in culture compared with controls (Figure 2c). MiR-9 expression levels upon ectopic expression were within a physiologic range, as miR-9 expression is 302-fold higher in MLL-rearranged patients as compared with t(8;21) patients (Supplementary Figure 3a). In the growth competition assay, GFP^+ miR-9-transduced cells were outcompeted by $mCherry^+$ miR-SC-transduced control cells, while the GFP^+ miR-SC-transduced cells were not (Figure 2d). The colony forming capacity showed a twofold reduction (Figure 2e). This growth reduction by miR-9 was not due to an increased rate of apoptosis or cell death (Figure 2f). However, this was not the case for miR-582, where the decrease in the number of viable cells was clearly caused by the induction of apoptosis (Figures 1b and 2f).

The reduction of cell growth was in part attributed to monocytic differentiation of the leukemic cells in conjunction with calcitriol. This was evident by the appearance of miR-9 transgenic cells with a lighter cytoplasm and uneven cell membranes exhibiting ruffles and protrusions. In addition to that, an enhanced expression of CD11b and CD14 was detected in comparison with the control cells in culture (Figure 2g-h). The induction of monocytic differentiation and reduction of cell growth by miR-9 was also observed in another t(8;21) cell line, SKNO-1, but not in THP-1 and NB4 (with t[15;17]) cells (Figure 2i). MiR-9 is marginally expressed in t(8;21) KASUMI-1 and SKNO-1 cells and lowly expressed in t(15;17) NB4 cells, while it is highly expressed in THP-1 cells (Supplementary Figure 4a). MiR-9 repression using a lentiviral sponge system conferred a growth advantage to SKNO-1 cells and growth disadvantage to THP-1 and NB4 cells (Supplementary Figure 4b-d). Thus, miR-9 blocks proliferation and induces differentiation in t(8;21) positive cells but not in *MLL*-rearranged and t(15;17) positive cells.

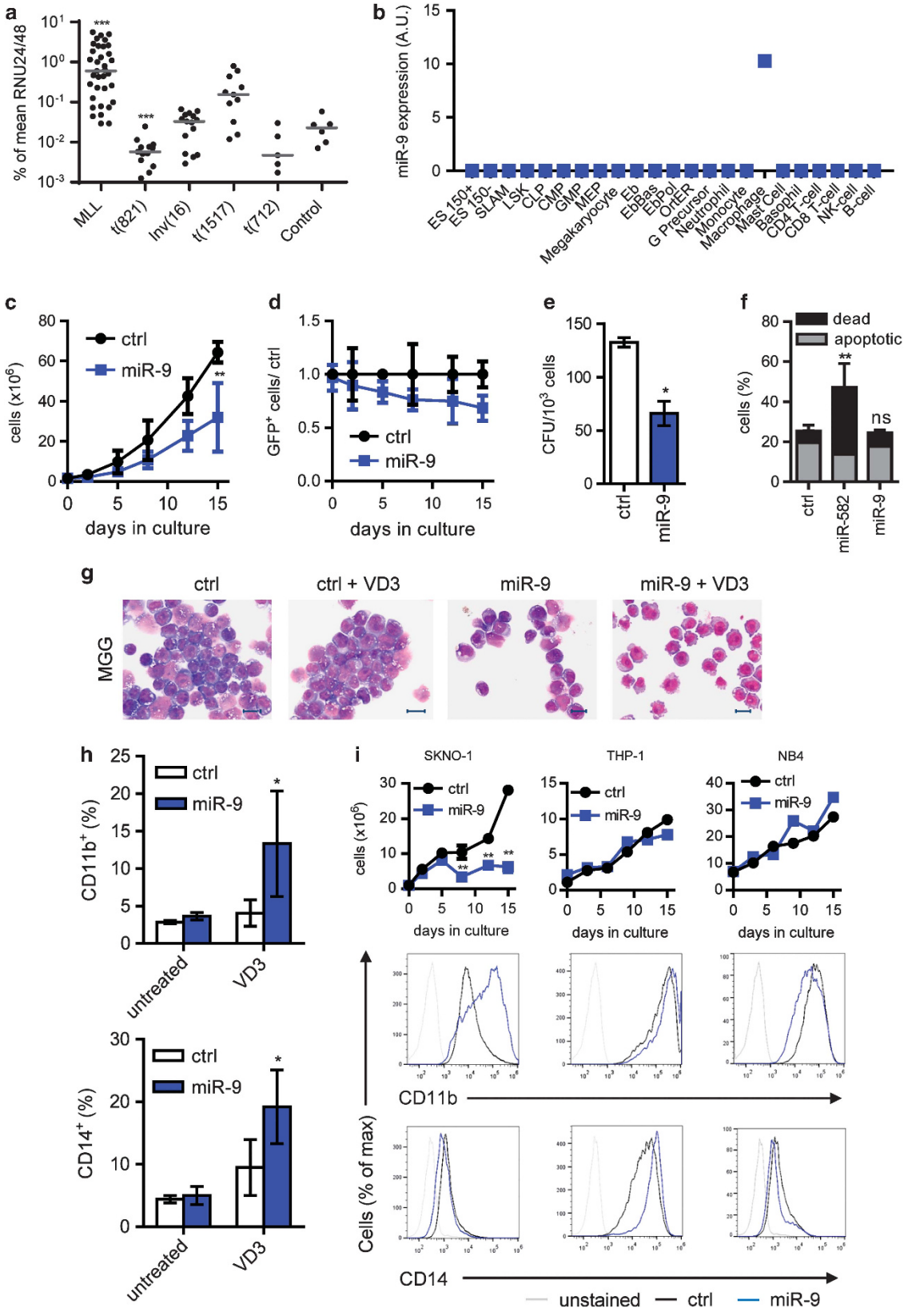
MiR-9 exerts a tumor-suppressive function in primary t(8;21) leukemic cells *in vitro* and in xenotransplantation models *in vivo*

To investigate if those cell-context specific effects are restricted to cell lines or apply globally to t(8;21) AML, we ectopically expressed miR-9 in primary leukemic blasts using the same lentiviral system. As in the cell lines, miR-9 suppressed leukemic growth and colony forming capacity of t(8;21) AML samples ($n = 2$; Figure 3a). On the other hand, the opposite effect was observed in *MLL*-rearranged samples ($n = 3$; Figure 3b). The suppression of leukemic growth in t(8;21) AML was accompanied by the induction of monocytic genes (Supplementary Figure 5a).

Using two independent xenotransplantation models, we could also prove the tumor-suppressive function of miR-9 *in vivo*. First we performed a competitive transplantation assay, where NRG mice were intrafemorally transplanted with miR-9- or miR-SC-transduced KASUMI-1 cells (GFP^+) together with untransduced competitors (GFP^-; GFP^+:GFP^- ratio 2:1; Figure 3c). While the

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GFP$^+$:GFP$^-$ ratio of 2:1 was mainly unchanged in the bone marrow of non-silencing control recipients after 28 days, it was reduced in the miR-9-recipient mice (Figure 3d). Thus, miR-9-transduced KASUMI-1 cells were outcompeted by their untransduced competitors. Furthermore, we could confirm the induction of monocytic differentiation of t(8;21) KASUMI-1 cells *in vivo*



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(Figure 3e and f). Similarly, the tumor size of nude mice, subcutaneously injected with transduced KASUMI-1 cells, was reduced by miR-9 (Figure 3g). Thus, miR-9 acts as a tumor suppressor in t(8;21) AML *in vitro* and *in vivo*.

MiR-9 overexpression in CD34$^+$-HSPCs and *MLL*-rearranged AML blasts impairs granulocytic and monocytic differentiation

To test if the effects of miR-9 are restricted to t(8;21) leukemic cells or if miR-9 generally induces myelomonocytic differentiation, we transduced and evaluated human CD34$^+$-HSPCs and *MLL*-rearranged AML blasts. Monocytic differentiation was suppressed in *MLL*-rearranged AML samples, as indicated by the reduced expression of monocytic genes and the round morphology of the cells (Supplementary Figure 5a and b).

During granulocytic *in vitro* differentiation of CD34$^+$-HSPCs, the miR-9-transduced CD34$^+$-HSPCs ceased to proliferate (Figure 4a). Accordingly, the granulocytic differentiation was diminished, as quantified by the fraction of CD13$^+$/CD15$^+$ granulocytes by flow cytometry (Figure 4b). During the monocytic *in vitro*

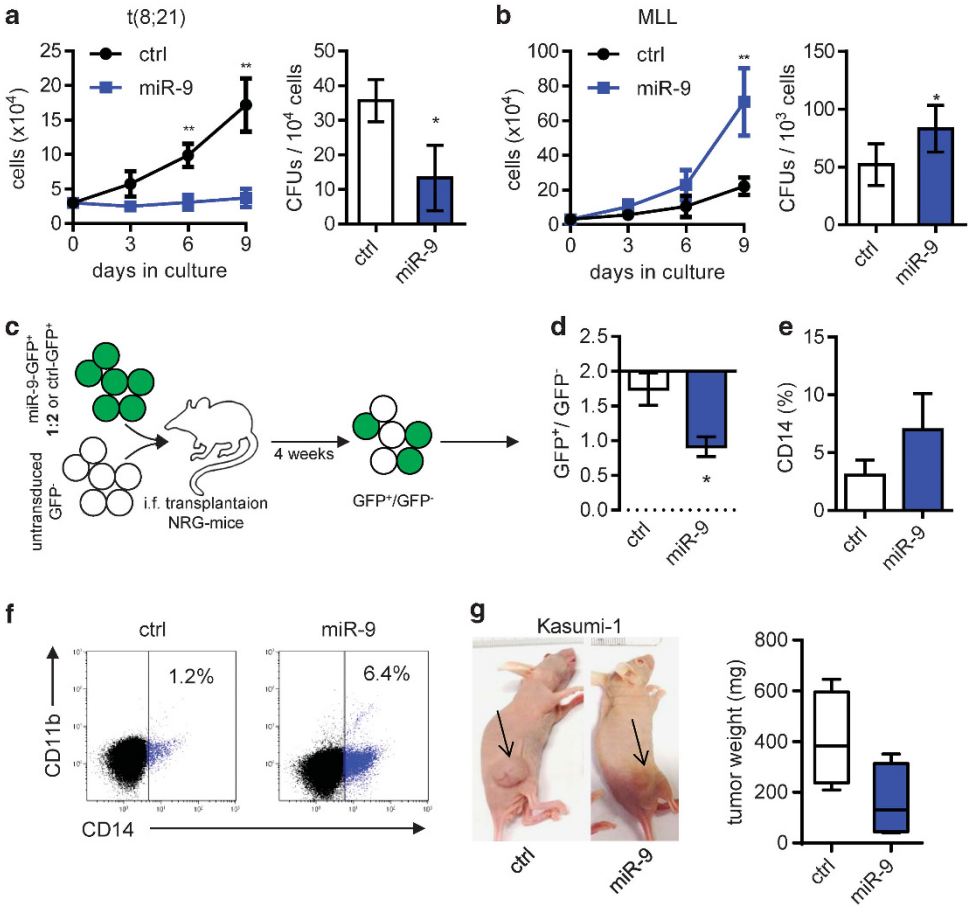


Figure 3. MiR-9 exerts a tumor-suppressive function in primary t(8;21) leukemic cells *in vitro* and in xenotransplantation models *in vivo*. (**a** and **b**) Cell counts (left diagram) and number of CFUs in methylcellulose-based colony-forming assays (right diagram) of miR-9 or miR-SC (ctrl)-transduced t(8;21) (**a**) or *MLL* rearranged (**b**) AML patient blasts ($N=2$ and $N=3$, respectively). (**c**) Scheme illustrating the experimental setup of the competitive xenotransplantation assay. (**d**) Ratio of GFP$^+$ transduced vs GFP$^-$ untransduced KASUMI-1 cells in NRG mice after 28 days following i.f. transplantation. (**e**–**f**) Percentage of CD14$^+$ (**e**) and representative flow cytometry plots (**f**) of GFP$^+$-transduced KASUMI-1 cells in the same mice as in (**d**). (**g**) Photographs (left) and tumor weights (right) of nude mice subcutaneously injected with miR-9 or miR-SC (ctrl) transduced KASUMI-1 cells after 6 weeks. (**d**–**g**) Data are presented as mean ± s.d. of $N=5$ mice per group (*$P<0.05$; **$P<0.01$).

Figure 2. Mir-9 suppresses proliferation and induces differentiation in KASUMI-1 cells. (**a**) Expression of miR-9-5p as measured by TLDA. CD34$^+$-HSPCs were used as a control. Expression is significantly higher in *MLL*-rearranged AML (Log_2-fold change = 6.98, $P<0.0001$) and significantly lower expressed in t(8;21) AML (Log_2-fold change = −7.58, $P<0.0001$) when compared with all other AML patients. (**b**) Expression of miR-9 in the indicated hematopoietic stem and/or progenitor cell compartments and mature blood cells.[31] (**c**) Total cell counts of miR-9 or miR-SC (ctrl) transduced KASUMI-1 cells. (**d**) Growth competition assay. The fraction of GFP$^+$ miRNA-transduced cells at indicated time points of culture is shown in relation to miR-SC (ctrl). (**e**) Number of CFUs in methylcellulose-based colony-forming assays of miR-9 and miR-SC (ctrl) transduced cells. (**f**) Percentage of apoptotic (Annexin V$^+$/7-AAD$^-$) and dead (7-AAD$^+$) cells for miR-9, miR-582 and miR-SC (ctrl) transduced KASUMI-1 cells measured by flow cytometry after 3 days of culture. (**g**) Representative microscopic images of May–Grünwald–Giemsa (MGG) stained cytospins (scale bars, 50 μm) of miR-9 or miR-SC transduced KASUMI-1 cells with and without VD3 treatment. (**h**) Percentage of CD11b$^+$ (upper graph) and CD14$^+$ (lower graph) KASUMI-1 cells as determined by flow cytometry. (**i**) Growth and differentiation in SKNO-1, THP-1 and NB4 cells. Upper panel, total cell counts of miR-9- or miR-SC-transduced untreated cells; lower panels, representative histograms of normalized fluorescence intensities from flow cytometric staining of indicated surface markers in SKNO-1 and THP-1 cells treated with VD3 and NB4 cells treated with ATRA. (**c**–**i**) Data are presented as mean ± s.d. of replicates of two to four independent experiments (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

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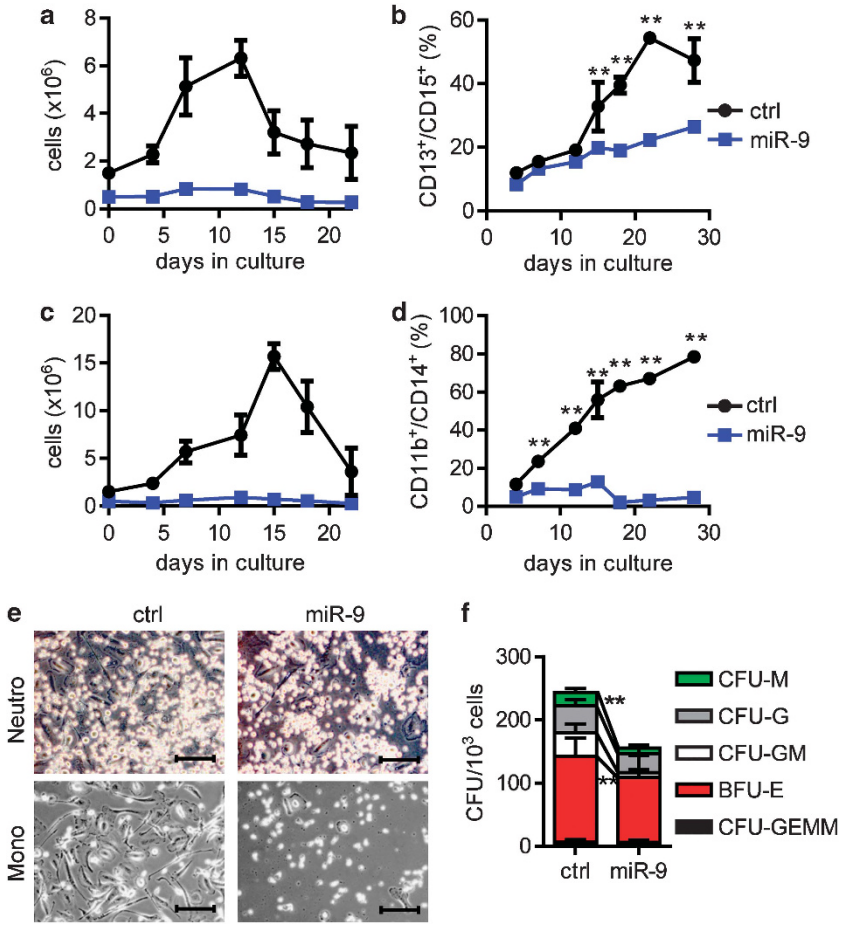


Figure 4. MiR-9 blocks *in vitro* cell growth and differentiation of HSPCs. (**a**, **c**) Total cell counts of miR-9- or miR-SC-transduced (ctrl) HSPCs during neutrophilic (**a**) and monocyctic (**c**) *in vitro* differentiation. (**b**, **d**) Percentage of $CD13^+/CD15^+$ cells during neutrophilic (**b**) and $CD11b^+/CD14^+$ cells during monocytic (**d**) *in vitro* differentiation. (**e**) Phase contrast microscopic images of *in vitro* differentiated HSPCs (scale bars, 200 μm). (**f**) Number of CFUs in methylcellulose-based colony-forming assays of miR-9 and miR-SC-transduced HSPCs. (**a**–**f**) Data are presented as mean ± s.d. of replicates from one of two independent experiments (**$P < 0.01$).

differentiation, the number of cells was 20-fold lower on day 15 (Figure 4c) and only few $CD11b^+/CD14^+$ monocytes were detected in culture (Figure 4d). The block in proliferation and differentiation was also apparent in phase contrast microscopy (Figure 4e). Especially during monocytic differentiation no fusiform, adherent macrophages were seen. In the CFU assay the number of monocytic and mixed granulocytic–monocytic CFUs was significantly reduced (Figure 4f and Supplementary Figure 6a and b).

Thus, in contrast to t(8;21) AML, miR-9 blocks myelomonocytic differentiation in HSPCs and *MLL*-rearranged AML. However, similar to t(8;21) AML and in contrast to *MLL*-rearranged AML, proliferation of HSPCs was blocked.

MiR-9 exerts its effects by targeting the *LIN28B*/let-7/*HMGA2* axis

To identify target genes which might explain these controversial findings, we performed global gene expression profiling in miR-9- and miR-SC-transduced $CD34^+$-HSPCs and KASUMI-1 cells. In total, 4712 genes were downregulated after miR-9 overexpression in both cell types. Overall, 93 genes out of 219 predicted miR-9 target genes were downregulated (Figure 5a and Supplementary Table 2). Among those genes were known miR-9 targets such as *CDH1*, *NFKB1*, *BACE1* and *REST*.

The transcription factor *MAF* acts in concert with *MAFB* as the two master regulators of macrophage development.[32,33] We could demonstrate an intronic miR-9 binding site in the *MAF* unspliced transcript as being responsive to miR-9 binding in reporter assays (Supplementary Figure 6c). This intron can be retained in *MAF* isoform B (isoB). Both isoforms were strongly repressed during *in vitro* monocytic differentiation (Supplementary Figure 6d). Repression of ten miR-9 target genes – downregulated in the microarray analysis – in transduced HSPCs was validated by RT-qPCR (Supplementary Figure 6e). Among those are *SHC1*, a mediator of SCF/Kit signaling, the oncogene *ATF1*, *PAK4* involved in inhibiting apoptosis and regulating cell motility, *PRUNE* and *CDH1* that play a role in cell migration. These genes give a possible explanation to the block in growth and differentiation and might be responsible for the drastic morphological change in cells.

Expression of High Mobility Group A2 (*HMGA2*) and lin-28 homolog (C. elegans) (*LIN28B*) has been correlated with leukemogenesis and the alteration of hematopoietic differentiation.[34,35] Both genes are predicted targets of miR-9 and were downregulated upon ectopic expression of miR-9 in both $CD34^+$-HSPCs and KASUMI-1 cells (Figure 5a and b). In addition to PCR validation, downregulation of HMGA2 and LIN28B was confirmed by western blotting in KASUMI-1 cells, but not in *MLL*-rearranged patient blasts (Figure 5c and Supplementary

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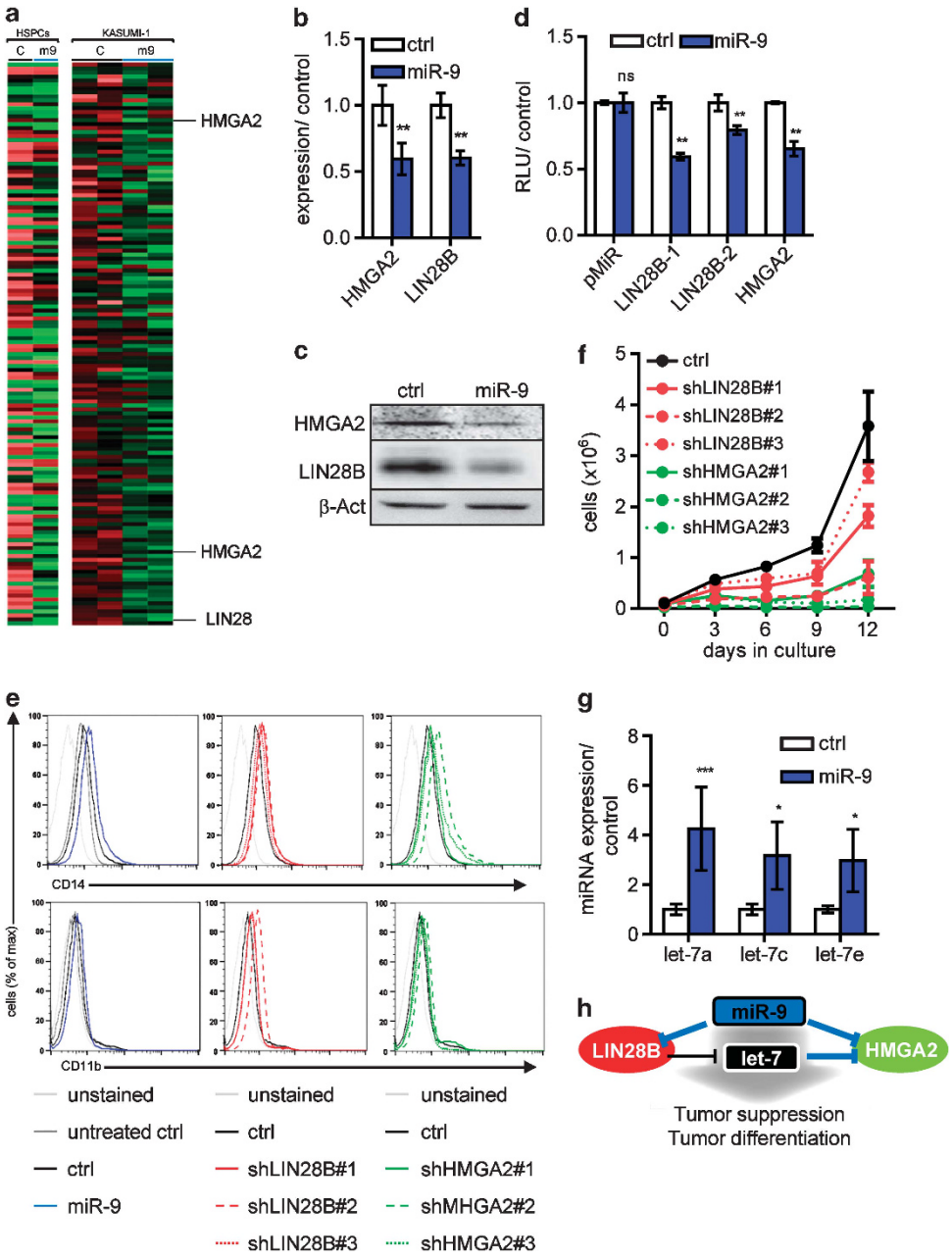


Figure 5. *LIN28B* and *HMGA2* are miR-9 target genes. (**a**) Heatmap displaying 93 validated and predicted miR-9 target genes downregulated in miR-9-transduced (m9) HSPCs ($n = 1$) and KASUMI-1 cells ($n = 2$) compared with the miR-SC-transduced controls (C), respectively; indicated are probe sets for *HMGA2* and *LIN28B*. (**b**) RT-qPCR of miR-9 target genes in KASUMI-1 cells. (**c**) Western blot of miR-9 target genes in miR-9- and miR-SC (ctrl)-transduced KASUMI-1 cells. (**d**) Luciferase reporter assay with indicated 3′UTR fragments in 293T cells. Data are presented as mean ± s.d. of replicates of five independent experiments (**$P < 0.01$). (**e**) Flow cytometry for CD11b and CD14 markers of miR-9-, miR-SC (ctrl), shLIN28 (#1-3)- and sh*HMGA2* (#1-3)-transduced KASUMI-1 cells. Except for untreated control each sample was treated with 50 nM VD3. (**f**) Number of viable cells of control- or shRNA-transduced KASUMI-1 cells determined by luminescence cell viability assay. (**g**) RT-qPCR of let-7 isoforms in miR-9- and miR-SC (ctrl)-transduced KASUMI-1 cells. (**h**) Molecular model of miR-9 anti-tumor mechanism. MiR-9 inhibits both *LIN28B* and *HMGA2*, additionally a double-negative feedback reliefs let-7 from *LIN28B* suppression and increases reduction of *HMGA2* and tumor suppression, overall resulting in enhanced tumor differentiation. (**b**, **f**, **g**) Data are presented as mean ± s.d. of replicates of two to four independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Figure 7a). *HMGA2* and *LIN28B* were validated as direct targets by 3′UTR luciferase reporter assays (Figure 5d).

To investigate whether repression of those two oncogenes at least in part mediates the effects of miR-9 on t(8;21) AML cells, we knocked down their expression using three independent shRNAs. Knockdown efficiency was confirmed by RT-qPCR (Supplementary Figure 7b and c). When shRNA-transduced KASUMI-1 cells were treated with calcitriol, we observed for each shRNA-construct a pronounced induction of CD11b or CD14 (Figure 5e). While knockdown of *LIN28B* more strongly enhanced expression of CD14, knockdown of *HMGA2* elevated CD11b more efficiently (Figure 5e). Accordingly, cell viability measurement over time revealed a growth disadvantage for all six shRNA constructs (Figure 5f). Likewise, colony-forming capacity was reduced by knockdown of the two genes (Supplementary Figure 7d). Thus, single knockdown of *LIN28B* and *HMGA2* was sufficient to partly

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recapitulate the miR-9 phenotype in KASUMI-1 cells; that is, induction of monocytic differentiation and suppression of leukemic growth.

LIN28B has been shown to suppress let-7 processing.[36] Interestingly, let-7 is a well-known tumor suppressor[37] and exerts its function at least in part by inhibiting the oncogene *HMGA2*.[38] To test whether this axis is involved in miR-9-mediated suppression of leukemic growth, we quantified expression of mature let-7 homologs let-7a, let-7c and let-7e by RT-qPCR. All three let-7 homologs were upregulated upon miR-9 expression in KASUMI-1 cells (Figure 5g).

Collectively our data demonstrates that miR-9 acts as a growth suppressor and differentiation inducer in t(8;21) AML by inhibiting *HMGA2* and *LIN28B* (Figure 5h). The two target genes are connected via let-7 miRNAs, which accumulate upon down-modulation of *LIN28B*, further inhibiting *HMGA2* and additionally feeding into miR-9 anti-tumor propensities.

DISCUSSION

Here we describe a large scale miRNA expression profiling study in pediatric AML, which revealed specific miRNA expression patterns in *MLL*-rearranged AML, t(8;21) positive AML, t(15;17) positive AML, and inv(16) positive pediatric AML. We combined this expression profiling with a functional characterization of seven candidate miRNAs/miRNA clusters in three cell lines. This led to the discovery that miR-9 suppresses leukemic growth while inducing monocytic differentiation in t(8;21) AML *in vitro* and *in vivo*. This growth inhibitory function was also evident in normal $CD34^+$-HSPCs while differentiation induction was restricted to t(8;21) leukemic cells. Several predicted targets were downregulated upon forced miR-9 expression in cell lines and primary patient samples. We showed that miR-9 exerts its effects in cooperation with let-7 by repressing the oncogenic *LIN28B*/*HMGA2* axis.

As miR-9 is encoded in three locations in the human genome and is highly conserved among species, it seems that a tightly controlled miR-9 expression is of great importance for cellular integrity. In our cohort of pediatric AML samples, miR-9 expression was significantly lower in t(8;21) positive AML and significantly higher in *MLL*-rearranged AML as compared with all other AML. In contrast to other studies showing high expression of miR-9 as a predictor of poor survival in adult AML,[8] miR-9 expression was not an independent prognostic factor in multivariate analysis in pediatric AML (data not shown). Nevertheless, we clearly showed that ectopic expression of miR-9 in AML cell lines and primary patient samples with t(8;21) conferred a tumor-suppressive phenotype and induced differentiation along the monocytic lineage. Our results are in accordance with a previous study showing that miR-9 releases the differentiation block of oncogenic *EVI1*.[39] Hence, the findings are clinically relevant since substances or genetic modulators that stop leukemic growth by releasing the differentiation block of leukemic blasts, are warranted in leukemia therapy.[40,41] For example, in acute promyelocytic leukemia, survival increased by introducing ATRA, which specifically induces differentiation in blasts harboring the fusion protein PML-RARα.[42] MiR-9 is exclusively expressed in macrophages during normal hematopoietic differentiation.[31] Therefore, we hypothesize that elevating the expression of miR-9 could provide a specific treatment option for t(8;21) AMLs, but not for *MLL*-rearranged AML, where miR-9 exerts oncogenic effects[43] (Figures 2 and 3).

By interfering with the oncogenic *LIN28B*/let-7/*HMGA2* axis, miR-9 can achieve its tumor-suppressive properties (Figure 5). The developmentally regulated RNA-binding protein *LIN28B* belongs to the group of factors that can be used for reprogramming somatic cells into pluripotent stem cells[44] and is overexpressed in various types of cancer.[36] *LIN28A* and *LIN28B* selectively repress expression of let-7 miRNAs.[45–48] The let-7 miRNA family members act as tumor suppressors by repressing oncogenes including *RAS*, *MYC* and *HMGA2*. In addition to that many tumors are characterized by the downregulation of multiple let-7 miRNAs.[49] In our study, ectopic expression of miR-9 led to downregulation of *LIN28B* and, as a consequence, to upregulation of three tested let-7 family members (Figure 5g). *HMGA2* codes for a small non-histone, chromatin-associated protein that modulates transcription by altering the chromatin architecture[50,51] and serves as a target of let-7 and miR-9.[38] In analogy to *LIN28*, *HMGA2* is expressed in undifferentiated proliferating cells during embryogenesis and in several benign and malignant tumors.[52] In many of these tumors, a truncation of the human *HMGA2* disrupts the binding and negative regulation by let-7, resulting in oncogenic transformation.[53] Thus, miR-9 inhibits two well characterized oncogenes, *LIN28B* and *HMGA2*, while restoring the expression of the linked tumor suppressor let-7. In concert, let-7 and miR-9 can even achieve a greater knockdown of oncogenic *HMGA2* (Figure 5h). This may release leukemic cells from their differentiation block and stop their growth.

Alternatively, downregulation of *LIN28B* and *HMGA2* in HSPCs might prevent the initiation of a differentiation program. In normal HSPCs, this failure in the induction of myelomonocytic differentiation upon miR-9 expression is fortified by the concordant repression of *MAF*, an essential transcription factor for myelomonopoiesis and another target of miR-9. We demonstrated that an intronic miR-9 binding site is responsible for miR-9-mediated downregulation of *MAF* isoforms during *in vitro* monocytic differentiation of normal HSPCs. Moreover, a set of miR-9 target genes, such as *ATF1*, *PAK4*, *PRUNE* and *CDH1*, associated to cell motility and migration, are repressed by ectopic miR-9 expression (shown by microarray analysis and RT-qPCR validation), thus, restricting a key function of terminally differentiated myelomonocytic cells. In contrast, in adult AML Senyuk *et al.* demonstrated that miR-9 not only induced myelomonocytic differentiation of malignant hematopoietic cells but also of normal murine Lin^--HSPCs.[39] Thus despite the overall concordance between the studies there are some differences, which might be attributed to different vectors systems (lentivirus vs retrovirus), resulting in different expression levels of miR-9, and different cell contexts (human $CD34^+$-HSPCs vs murine Lin^--HSCPs) tested.

In conclusion, by performing miRNA expression screening in pediatric AML, we gained novel insights in the biology and development of this disease. The identification of miR-9 as a novel tumor suppressor and differentiation inducer in t(8;21) AML opens the avenue for future therapeutic options.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

SE, KH, FE, RJ, FD, and MEK performed all *in vitro* and *in vivo* experiments and analyzed data. JEK-K, LV, AAD-vO. performed expression studies and analyzed data. SE designed experiments and wrote the manuscript. KH and JEK-K wrote the manuscript. MEK revised the manuscript. JHK designed and supervised the study, analyzed data and wrote the paper. MMvdH-E designed and supervised

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the study and wrote the manuscript. DR, JS, AB and VdH contributed materials and clinical data and revised the manuscript. DR, CMZ, MLdB, MF and RP supervised the study and revised the manuscript.

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