miR-9/9*

in Myeloid Development and Acute Myeloid Leukemia

Katarzyna Nowek

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miR-9/9* in Myeloïde Differentiatie
en Acute Myeloïde Leukemie

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To all ladies for the gentle strength within them.

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1

GENERAL INTRODUCTION

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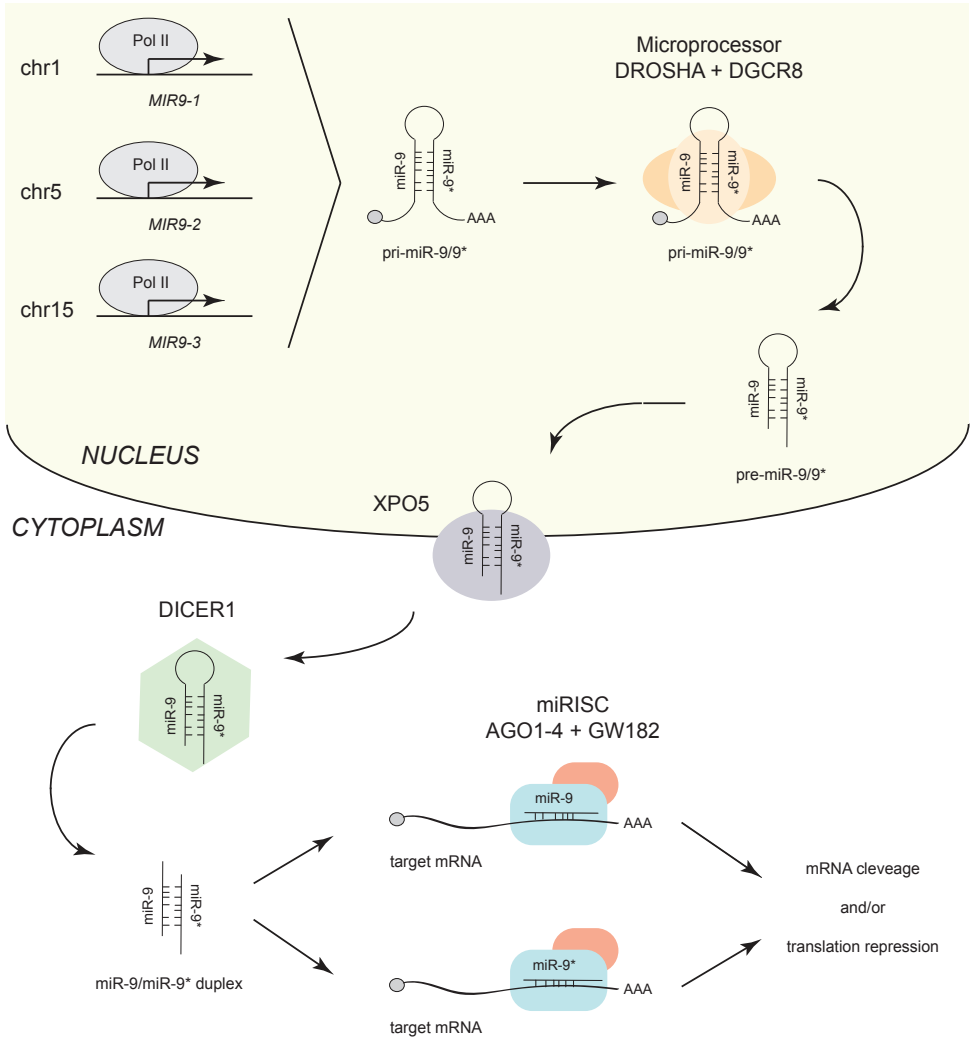

Figure 1. Schematic overview of canonical miRNA biogenesis pathway demonstrated by miR-9/9*. miRNA genes are usually transcribed by RNA polymerase II (Pol II) as long primary transcripts (pri-miRNAs) that are then cleaved by Microprocessor complex (DROSHA and DGCR8) to release a smaller precursor miRNA (pre-miRNA). Pre-miRNA is transported from nucleus to the cytoplasm by exportin 5 (XPO5) and further processed by DICER1. miRNA/miRNA* duplex, generated by DICER1, is subsequently loaded onto AGO protein, unwinded, and one strand of the mature miRNA becomes a part of miRNA-induced silencing complex (miRISC). miRISC regulates target gene expression by transcript destabilization and/or translational repression. Chr, chromosome.

GENERAL INTRODUCTION

An adult human organism consists of trillions of cells that collectively constitute its complex tissues and organs. The development of those cells depends on activation or suppression of specific gene programs that regulate cell division, differentiation and cell death. The coordinated regulation of those programs occurs through among others transcriptional and post-transcriptional mechanisms. It has become apparent that the cellular mechanisms controlling normal and abnormal hematopoiesis can be profoundly regulated by microRNA's. MicroRNAs (miRNAs) belong to the family of small non-coding RNAs, together with siRNAs and piRNAs, that post-transcriptionally suppress gene expression.[1] By base pairing with their target mRNAs, miRNAs function as a guide for other factors that inhibit translation and/or induce mRNA decay.[2] Considering that more than 1000 distinct miRNA genes have been identified and more than 60% of human protein-coding genes are thought to be under the control of miRNAs, these small molecules have a role not only in physiological gene regulatory networks but they may also have an important role in the dysregulation of these networks in human disease.[3-5] This thesis is focused on the functional significance of microRNA's in normal hematopoiesis and the pathobiology of acute myeloid leukemia (AML).

1. MIRNAS

1.1. miRNA biogenesis

miRNA genes

miRNA gene sequences are located within various genomic contexts, such as intronic and exonic regions of other transcripts.[6] Several miRNA loci may constitute a policistronic transcription unit, where a single transcript yields multiple mature miRNAs that are co-transcribed.7 The expression of individual miRNAs can be post-transcriptionally regulated to provide robustness and versatility. Some miRNAs are expressed from multiple paralogous loci in a given genome.[7] This results in increased redundancy and reflects functional importance of such miRNAs.

The precise location of miRNA promoters have not yet been mapped for most miRNA genes.[8] Additionally, intronic miRNAs may have their own promoters or share them with their host genes. The transcription of the majority of miRNA genes is carried out by RNA polymerase II and results into formation of long primary (pri-miRNAs) transcripts (**Figure 1**).[6,8] Pri-miRNA transcription can be deregulated and can promote cancer initiation.[9] This occurs when the pri-miRNA genomic region is deleted, amplified or translocated, but also when there is an aberrant transcription factor activity.

Pri-miRNA processing

The long pri-miRNAs (typically with a length over 1 kb) are cleaved by the Microprocessor to release smaller (around 65 kb) hairpin-shaped precursor miRNAs (pre-miRNAs) (**Figure 1**).[1] The Microprocessor is a complex that includes the double-stranded RNase III enzyme DROSHA and its essential cofactor DGCR8.[1º] The target recognition by miRNA is dependent on the 5' end of its mature sequence that spans from nucleotide position 2 to 7 and is called the seed sequence.[11] DROSHA determines the 5' terminus of a miRNA and controls its abundance and activity, e.g. by post-translational modifications or interactions with RNA-binding proteins that selectively interact with certain pri-miRNAs.[9,12,13] Following DROSHA processing, pre-miRNA is exported into the cytoplasm by exportin 5.[14] It has been suggested that exportin 5 may protect pre-miRNA from nucleolytic attack in the nucleus, thus influencing its abundance in the cell.

Pre-miRNA processing

In the cytoplasm, pre-miRNAs are processed by another RNase III enzyme, DICER1, to form small ~22 nucleotide duplexes that comprise the mature miRNA and a similar-sized fragment derived from the opposing arm called miRNA* (**Figure 1**).[6] DICER1 determines the size of miRNA. It has been proposed that it works as a 'molecular ruler' that binds, measures and cleaves a pre-miRNA.[15] Genetic mutations and deregulation of DICER1 levels may result in tumorigenesis.[15] Mutation hot spots in the RNase III domain cause defects in DICER1-mediated processing of the 5' side of the pre-miRNA hairpin (5p miRNA). This leads to the loss of 5p RNA, whereas processing of 3p RNA (derived from the 3' side of the pre-miRNA) remains unaltered.[16]

miRNA/miRNA* duplex processing

The miRNA/miRNA* duplex, generated by DICER1, is subsequently loaded onto AGO protein, unwinded, and one strand of the mature miRNA becomes a part of a ribonucleoprotein complex called miRISC (**Figure 1**).[6] Different miRNA duplexes may be preferentially loaded onto particular types of AGO proteins. At steady state, usually one of the strands from the duplex is retained, whereas another is peeled away and degraded. The most abundant (guide) strand is referred to as a miRNA and the less abundant (passenger) as a miRNA*. The guide strand that will be loaded onto AGO is determined based on thermodynamic stability of the two ends of the duplex and the first nucleotide sequence.[17,18] The strand with relatively less tightly paired 5' terminus and/or U at nucleotide position 1 is typically selected, and the other one is generally degraded. Alternative DROSHA processing that changes the miRNA duplex ends may result in 'arm-switching' and selection of less abundant passenger strand.[13] miRNA maturation and turnover is influenced by alterations in RNA sequence and/or structure, such as single nucleotide polymorphisms, miRNA tailing, RNA editing and methylation. Single nucleotide polymorphisms may alter miRNA specificity and affect processing.[19] Non-templated nucleotide addition to the 3' end of RNA (miRNA tailing) may block DICER1 processing and lead to miRNA decay or conversely it may increase biogenesis.[20] RNA editing and methylation interfere with DROSHA- or DICER1-mediated processing.[21,22] miRNA abundance may be regulated also via miRNA stability.[23] Several endoribonucleases have been shown to cleave and degrade selected miRNAs. Additionally, miRNAs can be stabilized or primed for degradation by binding to their mRNA targets. The mechanisms of target-mediated stability control remain unclear.

Beside the canonical biogenesis pathway outlined above, there are several non-canonical pathways that include differential generation of small precursor RNA and processing independent of DROSHA or DICER1.[24] However, the group of miRNAs that undergo non-canonical processing probably comprises no more than about 1% of conserved miRNAs.

1.2. miRNA function

miRNA-target interactions

A miRNA-induced silencing complex (miRISC) consists of the miRNA bound to AGO together with associated proteins (**Figure 1**). miRNA acts as a guide for recognition of mRNA targets that subsequently undergo translational repression and/or decay.[2] Target recognition occurs through partial base pairing of the miRNA seed sequence with the 3' untranslated region (3' UTR) of an mRNA or rarely the open reading frame (**Figure 2**).[25] Sites that are complementary to the miRNA seed are evolutionarily conserved and are called 'canonical' sites. These sites contain sequences that match to the seed but also may pair with position 8 and/or have adenine opposite position 1 of a miRNA. Canonical sites have the largest effect on mRNA target expression. However, more abundant miRNAs also have a large proportion of non-canonical sites.[26] Non-canonical binding sites have bulges in the miRNA seed region and are less effective in changing target levels.

Other factors that play a role in the target's response to miRNA activity include the localization of the target site within transcript. The miRNA target sites that have the strongest impact on the repression of target genes are located at the beginning or the end of long 3' UTRs.[27] Target sites in coding regions have a smaller influence. Another important factor is the accessibility of the target site, that relates to the energy that is required to acquire a single-stranded conformation.[28] Specific miRNA-target interactions are regulated by RNA-binding proteins that bind with sequence elements around the target sites and lead to mRNA stabilization or destabilization.[11,29] Additionally, multiple target sites, either for a single miRNA or for a combination of miRNAs, may result in cooperative downregulation and together profoundly impact the level of target suppression.[30] Finally, the response of one target may be influenced by the competition of miRNA binding with other targets.

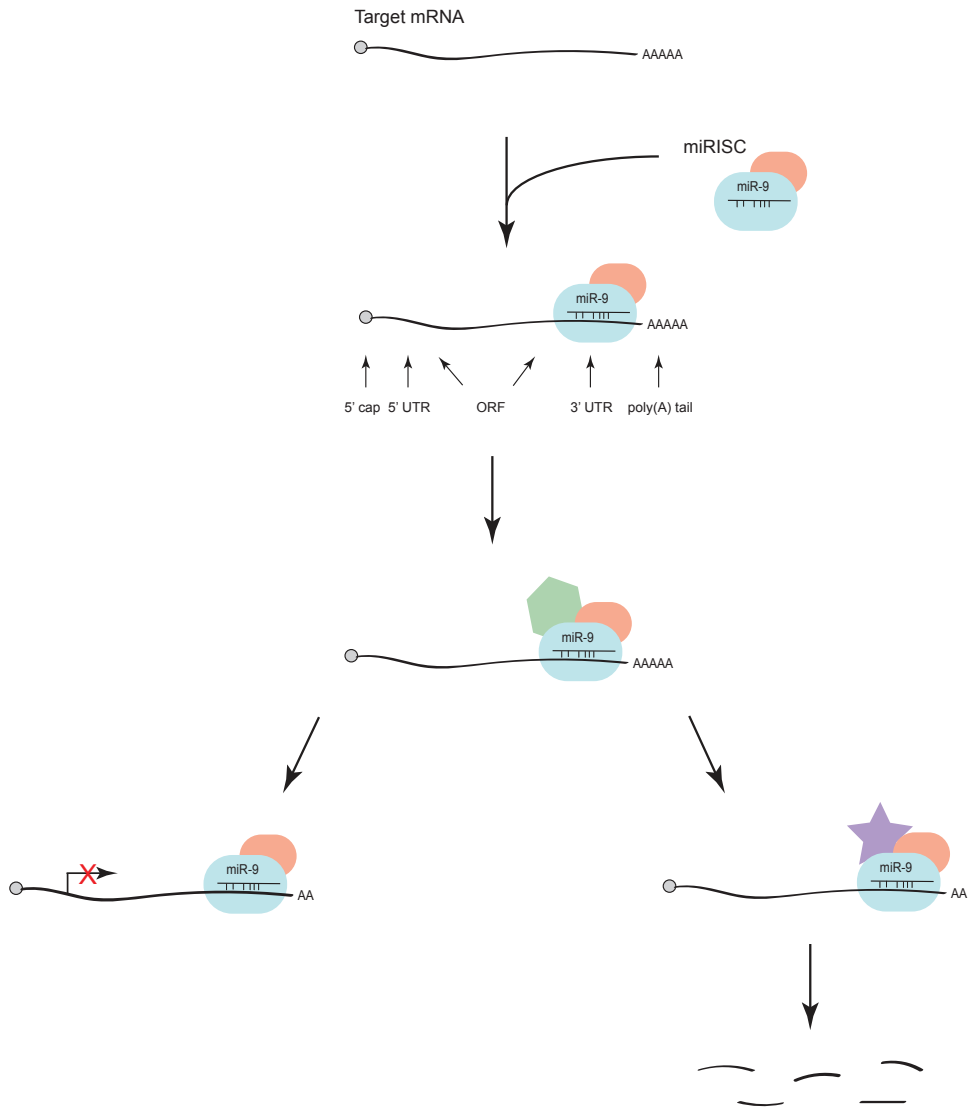

Figure 2. Schematic overview of a miRNA function demonstrated by miR-9. miRNA acts as a guide for miRISC for a recognition of mRNA targets by pairing to a binding site within the 3' untranslated region (3' UTR) of an mRNA or the open reading frame (ORF). This leads to translational repression and/or mRNA decay. Figure adapted from Huntzinger et al.[2]

miRNA-dependent target regulation

miRNA-target interaction ultimately results in a decrease in the level of target protein. Each miRNA can target hundreds of mRNAs that in combination may exert functional effects and change cellular phenotypes. miRNAs have been proposed to increase the precision of target gene expression by counteracting 'leaky' transcription, defining spatial domains of target expression and buffering transcriptional 'noise'.[31,32]

The abundance of miRNA targets is inversely correlated with the miRNA's ability to repress the expression of a specific gene. Several 'competing' RNAs are known to sequester miRNAs from their protein-coding targets.[29] These include pseudogenes, long non-coding RNAs and circular RNAs.[11] If the level of miRISC is less than the target mRNA or if these two are at similar concentrations, then competing RNA will have little effect on the mRNA target. However, if miRISC is initially in high excess compared to the target, then the expression of competing RNA can strongly upregulate the target.

In conclusion, miRNA establish thresholds in the levels of expression of their targets, reduce the variability of target gene expression between cells and regulate correlations between the expression of various targets within individual cell.[11]

2. MIRNA FUNCTION IN NORMAL MYELOID CELLS

2.1. Hematopoiesis and myeloid differentiation

Hematopoiesis is a hierarchical differentiation process in which hematopoietic stem cells (HSCs) undergo step-wise maturation into various types of blood cells (**Figure 3**).[33] During this process, HSCs lose their self-renewal and multilineage differentiation ability to develop into multipotent progenitors (MPPs) and subsequently give rise to lymphoid and myeloid progeny.[34] During lymphopoiesis, MPPs develop into common lymphoid progenitors (CLPs) and further into dendritic cells (DCs), natural killer cells (NKs), T cells and B cells. During myelopoiesis, MPPs differentiate into common myeloid progenitors (CMPs). CMPs give rise to myeloid lineage and develop into megakaryocyte/erythroid progenitors (MEPs) and granulocyte/monocyte progenitors (GMPs). MEPs further differentiate into erythrocyte precursors (ErPs) and red blood cells or megakaryocyte precursors (MkPs) and platelets , whereas GMPs into macrophages, granulocytes and DCs. Granulocytes consist of neutrophils, basophils and eosinophils.

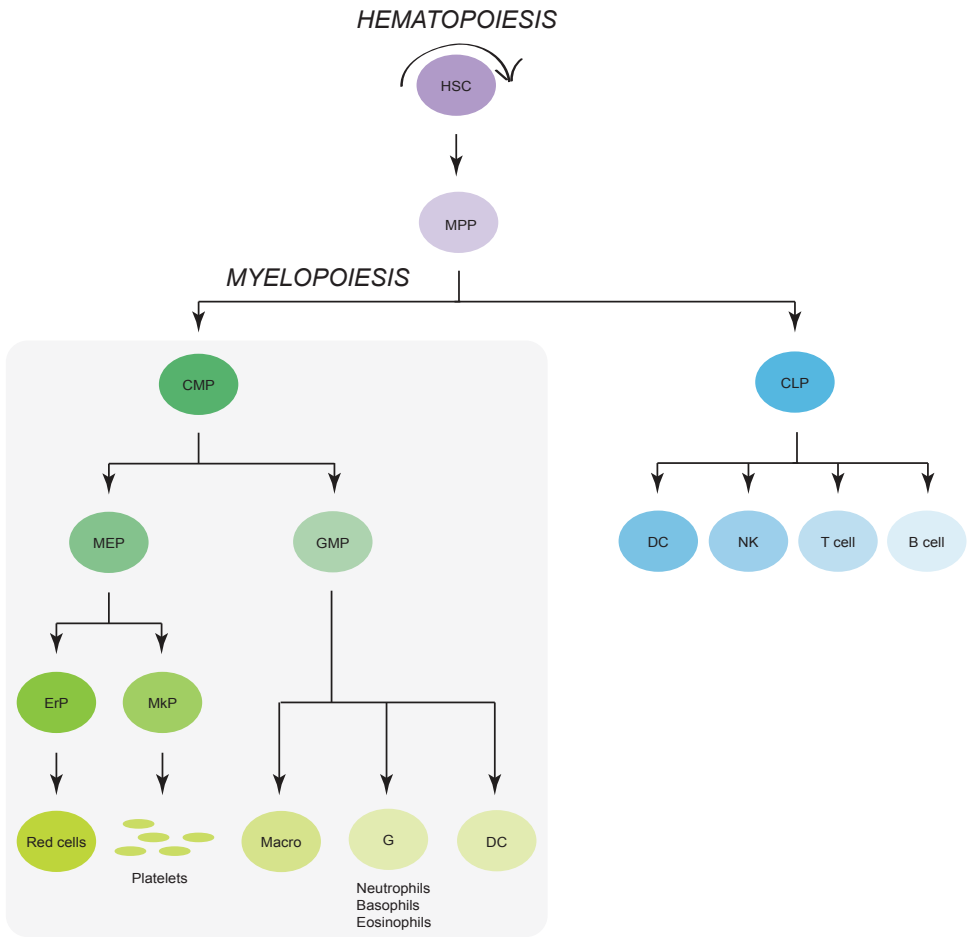

Figure 3. Schematic representation of hematopoiesis. Hematopoiesis is a hierarchical differentiation process in which hematopoietic stem cells (HSCs) undergo step-wise maturation into various types of blood cells. These cells further mature into multipotent progenitors (MPPs) and differentiate into common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). CLPs give rise to lymphoid lineage, i.e. dendritic cells (DCs), natural killer cells (NKs), T cells and B cells. CMPs give rise to myeloid lineage and develop into megakaryocyte/erythroid progenitors (MEPs) and granulocyte/monocyte progenitors (GMPs). MEPs further differentiate into erythrocyte precursors (ErPs) and red blood cells or megakaryocyte precursors (MkPs) and platelets, whereas GMPs differentiate into macrophages (Macros), granulocytes (Gs) and DCs. Gs consist of neutrophils, basophils and eosinophils. Figure adapted from Reya et al.[34]

2.2. miRNAs in normal myelopoiesis

The involvement of miRNAs in myeloid biology has been intensively studied.[35] They are components of regulatory feedback loops with various transcription factors important for myeloid development. miRNAs that have been reported to be involved in normal and malignant myelopoiesis together with their regulatory networks are summarized in **Table 1**. The examples of miRNA functions in normal myelopoiesis are given below for the three major players in myeloid development: miR-125 family, miR-155 and miR-223.

miR-125 family consists of miR-125a, miR-125b1 and miR-125b2.[36,37] These miRNAs have similar functions and exert a comparable effect on the hematopoietic phenotype. They are highly expressed in HSCs and promote the survival of this population by targeting pro-apoptotic factors.[36,38,39] Additionally, miR-125's have been reported to regulate HSC self-renewal and differentiation.[40] Their level of expression is regulated in a cell type-dependent manner by several transcription factors, such as NF-κB, p53, MYC, AKT1.[37] miR-125's negatively regulate granulocytic differentiation induced by granulocyte colony-stimulating factor (G-CSF).[37,41] They are also involved in negative regulation of inflammatory response by targeting tumor necrosis factor α (TNF-α) and, unusual for miRNAs, enhancing the stability of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inhibitor NKIRAS2.[37]

In most hematopoietic cells, miR-155 is expressed at low levels but it is rapidly upregulated in granulocyte-monocyte lineage during inflammatory stress, which probably occurs under the control of NF-κB and activator protein 1 (AP-1).[35,42-44] On the other hand, the expression of miR-155 is downregulated by signal transducer and activator of transcription 3 (STAT3) and protein kinase B (PKB/AKT1).[45,46] miR-155 impacts hematopoietic development by targeting transcripts of genes relevant to myeloid biology, e.g. PU.1 and CCAAT/enhancer-binding protein β (C/EBPβ),[44] and the function of mature immune cells by targeting genes involved in inflammatory pathways, e.g. SHIP1 and suppressor of cytokine signaling 1 (SOCS1).[46,47]

miR-223 is highly expressed in myeloid cells and its expression increases during granulocytic differentiation.[48] The miR-223 promotor is bound by nuclear factor 1 A-type (NF1A) and this interaction results in low expression levels of miR-223 in progenitor cells. During differentiation, C/EBPα binds to the same sites and increases miR-223 expression. Once expressed, miR-223 targets NF1A, turns off its repressor and thereby forms a positive-autoregulatory loop.[48] Upregulation of miR-223 promotes differentiation but miR-223 is not absolutely required for production of granulocytes *in vivo*. Additionally, miR-223 forms a negative-autoregulatory circuit with cell-cycle regulator E2F1 to promote an exit from the cell cycle and to contribute to differentiation.[49] It downregulates E2F1 expression, and conversely, E2F1 inhibits miR-223 transcription.

In conclusion, miRNAs are relevant players in hematopoiesis and specifically in myeloid development. They are components of crucial regulatory networks and they connect cellular pathways that influence hematopoietic cell fate decisions

3. MIRNAS IN ACUTE MYELOID LEUKEMIA

3.1. Acute myeloid leukemia

Acute myeloid leukemia (AML) is a form of cancer that is characterized by the accumulation of abnormally differentiated myeloid cells in the bone marrow (BM).[50] It has been proposed that impairment of differentiation and the proliferative advantage of hematopoietic cells are two key factors involved in the pathobiology of AML.[51] The considerable heterogeneity in cytogenetic and molecular abnormalities that are apparent in this disease is a unique feature of AML. The latter variations correlate with patient outcome and may inform treatment strategies.[50,52] Genes that are commonly mutated in AML and which normal function is disrupted fall into following categories: myeloid transcription factors, epigenetic regulators, tumor-suppressor and signaling genes. Recently, miRNAs have been recognized to regulate hematopoiesis and to contribute to leukemic development.

3.2. miRNAs in AML

In the past years, several studies have reported on deregulated expression of miRNAs in leukemia, and the relationship of patterns of deregulation with different cytogenetic and molecular subgroups in AML.[53,54] Specific miRNA signatures may correlate with different cytogenetic subtypes of AML. miRNA profiling has been proposed as a useful diagnostic tool.[55] Additionally, miRNAs may offer potential novel therapeutic agents for treatment of leukemia.[56] As a single miRNA can regulate multiple targets and signaling pathways, the use of miRNAs in human-cancer therapy might theoretically downregulate oncogenic effects. This justifies the efforts to explore the functional role of aberrantly expressed miRNAs in AML and their therapeutic influence on patient survival (**Table 1** and **2**). Some of the most frequently studied miRNAs in AML are discussed below in detail.

miR-125b is upregulated in AML with the highest levels in patients carrying t(2;11) and t(15;17).[40,53] Aberrant expression of miR-125b in normal hematopoietic progenitors blocks granulocytic differentiation through repression of core binding factor β (CBFβ) and leads to myeloproliferative disorder.[57] The effect of miR-125b on cell transformation is dose dependent and may result in development of different kinds of leukemia.[58,59] The critical targets of miR-125b in leukemogenesis are largely unknown but it has been proposed to regulate different components of p53 network.[60]

In patients with t(8;21) and cytogenetically normal AML, high level of miR-126 expression is associated with worse prognosis.[61,62] Both overexpression and knockout of miR-126 in leukemia stem cells (LSC) harboring AML1-ETO fusion protein result in enhanced leukemogenesis *in vivo* in mice.[61] miR-126 confers its effect likely through targeting ERBB receptor feedback inhibitor 1 (ERRFI1) and sprouty-related, EVH1 domain-containing protein 1 (SPRED1) when overexpressed, and through frizzled-7 (FZD7) when knocked out. It is the first example of a miRNA playing 2-faceted role in AML. Additionally, miR-126 was found to inhibit cell cycle progression and differentiation, and promote self- renewal and chemoresistance of LSC by targeting the PI3K/AKT/MTOR signaling pathway.[63] Targeting of miR-126 in LSCs resulted in depletion of LSC population *in vitro* and *in vivo*.[62,64]

miR-155 is upregulated in *FLT3*-ITD AML, where it is a part of transcription factor regulatory network involving several upstream regulators, e.g. JUN and runt-related transcription factor 1 (RUNX1), and two downstream targets, i.e. PU.1 and C/EBPβ.[65,66] Overexpression of miR-155 in HSC-enriched BM cells induces myeloproliferation without progression to overt AML.[47] The effect of miR-155 may result from downregulation of its targets SHIP1 and C/EBPβ. Mice with global SHIP1 deficiency develop a myeloproliferative disorder that resembles the miR-155 phenotype and C/EBPβ is known to have a critical role in granulopoiesis. Downregulation of miR-155 in AML cells results in upregulation of SHIP1 and PU.1 and leads to monocytic differentiation and apoptosis.[67] Additionally, it prolongs the survival of mice transplanted with leukemic cells.

miR-223 regulates myeloid differentiation as part of the already mentioned autoregulatory feedback loops involving C/EBPα and E2F1.[48,49] Additionally, it negatively regulates granulocytic proliferation by targeting the transcription factor myocyte-specific enhancer factor 2C (MEF2C), which is upregulated in highly proliferative leukemic progenitors.[68] miR-223 is downregulated in patients with t(8;21), where it is targeted for epigenetic silencing by AML1-ETO.[69] The loss of miR-223 in murine HSCs and human cord-blood $CD34^+$ cells leads to the expansion of myeloid progenitors.[70,71]

Other miRNAs that contribute to AML development are the miR-17~92 cluster and miR-29 family. The miR-17~92 cluster is overexpressed in *MLL*-rearranged leukemia.[72] Its induced expression enhances the colony-forming capacity of progenitor cells in part through downregulation of p21, the inhibitor of cell cycle progression.[72,73] The miR-29 family may be upregulated, i.e., in cytogenetically normal AML, or downregulated, i.e., in patients with t(11q23) rearrangement.[54,65] Overexpression of miR-29a in hematopoietic progenitors enhances self-renewal and results in a myeloproliferative disorder that progresses to AML.[74] On the other hand, miR-29b that possesses the same seed region acts as a tumor suppressor by inducing apoptosis.[75] These opposite functions of closely related miRNAs indicate the complexity of studying miRNA function and its targets and the importance of these studies in a relevant cellular context.

Variable levels of miRNA expression in leukemic cells may predict treatment outcome in patients with AML (**Table 2**). Thus, particular miRNAs could potentially be useful for risk assessment of patient with AML (reference). Pediatric patients with AML with low serum levels of miR-370 show reduced overall survival (OS) and relapse-free survival (RFS).76 In adults with cytogenetically normal AML, low miR-155 and high miR-181a expression are associated with prolonged OS and higher complete remission (CR) rate.[77,78] Additionally, low miR-3151 is associated with better OS and disease-free survival (DFS).[79] Until today, miR-212 is the only microRNA which has been reported to predict for better OS and RFS independently of cytogenetic subtype of adult AML.[80]

Table 1. miRNAs involved in normal myeloid development and AML

miRNA[a]	Upstream regulators	Downstream targets	Function
miR-1-2	EVI1		Proliferation[97]
let-7 family	CXCR4, YY1	PBX2	Differentiation[98] Chemosensitivity[99]
miR-10a		KLF4, RB1CC1	Proliferation and apoptosis[100]
miR-15a		WT1	Proliferation[101,102] Enhanced differentiation[103]
miR-16	FLT3/ITD	Pim-1, WT1	Proliferation[101,102,104] Enhanced differentiation[103]
miR-17 precursor family (miR-17~92, miR-106a~363, and miR-106b~25)	C/EBPβ, HIF-1α	PHLPP2, p21, STAT3, SQSTM1	Differentiation[105,106] Proliferation and self-renewal[107]
miR-21	GFI1		Differentiation[108]
miR-24	AML1-ETO	MKP-7, (potentially BIM and Caspase 9)	Proliferation and differentiation[109] Apoptosis[110]
miR-26a		E2F7	Proliferation and differentiation[111]
miR-27a		PLAG1	TNF-related apoptosis-inducing ligand (TRAIL) sensitivity[112]
miR-29 family	NRF2, c-Myc, KIT, C/EBPα, DNA copy number variations	AKT2, CCND2, DNMTs, CDK6, SP1, KIT, FLT3, SKI, MLLT11	Proliferation, apoptosis and chemosensitivity[75,113-116] Proliferation, differentiation and self-renewal[74,115,117-119]
miR-30c	C/EBPα	Notch1	Differentiation[120]
miR-32		BIM	Apoptosis[121]
miR-34 family	Methylation, C/EBPα	CREB, PD-L1, E2F3, BMyb	Apoptosis[122] Proliferation and differentiation[123-125] Tumor-mediated immunosuppression[126]
miR-99a		CTDSPL, TRIB2	Proliferation and apoptosis[127]
miR-100		RBSP3	Proliferation and differentiation[128]
miR-125 family	NF-κB, p53, MYC, AKT1, NRF2	G-CSF signaling, TNF-α, NKIRAS2, CBFβ, p53 network	Self-renewal and differentiation[36,40,57] Proliferation, apoptosis and differentiation[38,39,41,58-60] Apoptosis and chemosensitivity[113]
miR-126		PI3K/AKT/MTOR signaling, ERRFI1, SPRED1, FZD7	Self-renewal, differentiation and chemosensitivity[61-63] Proliferation and apoptosis[64,129]
miR-133		EVI1	Chemosensitivity[130]
miR-139-5p	HDAC	EIF4G2	Proliferation and differentiation[131]
miR-139-3p		HuR	Proliferation[132]
miR-142-3p		IFN-γ signaling-related genes, gp130, C/EBPβ	Development and differentiation[133,134]
miR-145	p73		Differentiation[135]
miR-146a	PU.1	CXCR4	Proliferation, differentiation and chemosensitivity[136] Differentiation[137]
miR-150	KLF4		Proliferation and differentiation[138]
miR-155	STAT3, AKT1, AP-1, NF-κB,PU.1	SHIP1, SOCS1, PU.1, C/EBPβ	Proliferation and function of immune cells[44-47] Apoptosis and differentiation[66,67,137]
miR-181 family		MLK2, ATM, PRKCD, CTDSPL, CAMKK1, HMGB1, MCL1, Bcl-2, p27Kip1	Proliferation[139,140] Differentiation[141,142] Apoptosis and chemosensitivity[143,144]
miR-182	HDAC	RAD51	Homologous recombination repair and chemosensitivity[145]
miR-193 family	Methylation, AML1-ETO, HOTAIR	AML1-ETO, DNMT3a, HDAC3, CCND1, c-KIT, MDM2	Proliferation, apoptosis and differentiation[146-149]
miR-196 family	GFI1	ERG	Differentiation[108,150]
miR199a-3p		PRDX6, RUNX1, SUZ12	Proliferation[132]
miR-222/221	AML1-ETO	KIT	Proliferation and differentiation[151]
miR-223	NF1A, C/EBPα, E2F1, AML1-ETO	NF1A, E2F1, MEF2C	Proliferation and differentiation[48,49,68-71]
miR-299			Differentiation[152]
miR-370	Methylation, DNA copy number variations	NF1, FoxM1	Proliferation[153,154]
miR-424		PLAG1	TRAIL sensitivity[112]
miR-495		PBX3, MEIS1	Proliferation, apoptosis and self-renewal[155]
miR-511	MLL-AF9	CCND1	Proliferation and differentiation[156]
miR-638		CDK2	Proliferation and differentiation[157]
miR-663			Differentiation[158]
miR-3151	SP1/NF-κB	TP53	Proliferation, apoptosis and chemosensitivity[159]

[a] miRNAs that have been reported to be involved in normal and malignant myelopoiesis together with their regulatory networks.

Table 2. Prognostic significance of miRNAs in AML

miRNA	miRNA level	AML subtype	Prognostic relevance
Pediatric			
miR-370	low	All	Shorter OS and RFS[76]
Adult			
miR-155	low	Normal karyotype	Longer OS and higher CR[78]
miR-181a	high	Normal karyotype	Longer OS and higher CR[77]
miR-212	high	All	Longer OS and RFS[80]
miR-3151	low	Normal karyotype	Longer OS and DFS[79]

Abbreviations: OS, overall survival; RFS, relapse-free survival; CR, complete remission rate; DFS, disease-free survival.

Table 3. miR-9/9* in hematological malignancies

Type	Upstream regulators	Downstream targets	Function
Lymphoid malignancies			
Acute lymphoblastic leukemia	Methylation	FGFR1, CDK6	Proliferation and apoptosis[85]
Hodgkin lymphoma	CD99	PRDM1	Differentiation[83,87,88]
Multiple myeloma	HDAC	IGF2BP3	Chemosensitivity[89]
Waldenström macroglobulinemia		HDAC4, HDAC5	Proliferation, apoptosis and autophagy[90]
Myeloid malignancies			
Acute myeloid leukemia	AML1-ETO, MLL fusion proteins	UBASH3B/Sts-1, LIN28B/HMGA2 axis, RYBH, RHOH, HES1	Proliferation and differentiation[91,92] Proliferation, apoptosis and self-renewal[93,94]

4. MIR-9/9* IN HEMATOLOGICAL MALIGNANCIES

4.1. miR-9/9*

miR-9 and miR-9* (miR-9/9*) are two miRNAs that originate from the same precursor RNA and are highly conserved during evolution from flies to humans.[81] All vertebrate miR-9/9* orthologs have the identical mature sequence. In mammals, miR-9/9* are encoded by three genes: *MIR9-1*, *MIR9-2* and *MIR9-3*. These genes are located in humans on the chromosomes 1, 5 and 15, respectively (**Figure 1**). miR-9/9* are mainly expressed in the nervous system and they were first studied as regulators of neurogenesis.[82] Since 2008, the aberrant expression of miR-9/9* has been demonstrated in various types of human cancer and a growing number of studies reported on their functional versatility dependent on the origin of the cancer cell.[53,83,84] The high level of sequence conservation and the fact that miR-9/9* are encoded at three different positions in the human genome suggest their important function in development and disease.

4.2. miR-9/9* in lymphoid malignancies

In acute lymphoblastic leukemia (ALL), low miR-9 expression is associated with hypermethylation of *MIR9* gene family (**Table 3**, **Figure 4**).[85] This epigenetic downregulation leads to upregulation of predicted miR-9 and miR-9* targets, fibroblast growth factor receptor 1 (FGFR1) and cyclin-dependent kinase 6 (CDK6). FGFR1 and CDK6 are involved in cell proliferation and survival. Treatment with FGFR1 and CDK6 inhibitors suppresses the proliferation of ALL cells.[85] *MIR9* genes have been reported to be frequently methylated also in chronic lymphocytic leukemia (CLL) and overexpression of miR-9 decreased CLL cell proliferation.[86]

CD99 is a transmembrane glycoprotein that is implicated in cell migration, adhesion and differentiation (**Table 3**, **Figure 4**).[87] It is lowly expressed in Hodgkin/Reed-Sternberg cells of Hodgkin lymphoma (HL). CD99 inhibits the transcription of miR-9 and upregulates the direct miR-9 target: positive regulatory domain 1 (PRDM1/BLIMP-1).[83,87] PRDM1 is the master regulator of terminal B-cell differentiation. miR-9 is highly expressed in HL cells and its downregulation by CD99 overexpression or a direct knockdown, augments PRDM1 levels that triggers B-cell differentiation into plasma cells.[87] During normal B-cell development within the germinal centers, B cells closely interact with follicular DCs.[88] Only B cells that bind to these cells survive in the germinal centers and differentiate. It has been shown that direct cell-cell contact between follicular DCs and B cells leads to downregulation of miR-9 and upregualtion of PRDM1. This subsequently may promote B-cell differentiation.

In multiple myeloma (MM), insulin-like growth factor 2 mRNA binding protein 3 (IGF2BP3) stabilizes the expression of a cell surface glycoprotein CD44 that is involved in drug resistance of MM cells (**Table 3**, **Figure 4**).[89] Histone deacetylase (HDAC) inhibitors are promising novel chemotherapeutics in MM since they downregulate CD44 expression.

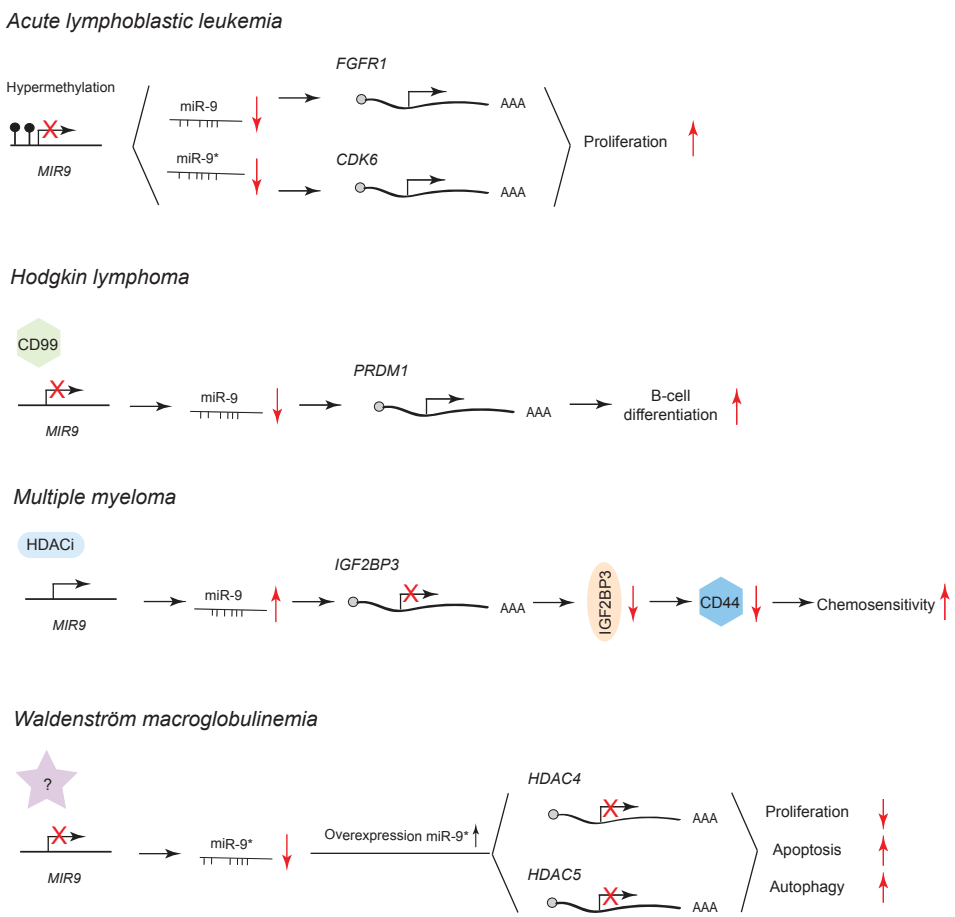

Figure 4. miR-9/9* function in lymphoid malignancies. In lymphoid malignancies, miR-9/9* have been reported to be differentially expressed, and to suppress or promote disease development depending on the cell of context.

HDAC inhibitors treatment leads to upregulatation of miR-9 and downregulation of its direct target IGF2BP3. Subsequent downregulation of CD44 sensitizes the resistant MM cell to lenalidomide treatment.

A passenger strand of miR-9, miR-9*, has been reported to have a tumor suppressive role in Waldenström macroglobulinemia (WM) (**Table 3, Figure 4**).[90] WM is a B-cell low-grade lymphoma characterized by the accumulation of B cells in the BM. miR-9* is lower expressed in WM CD19+ cells than in normal controls. Overexpression of miR-9* in WM cells inhibited the unbalanced HDAC activity by downregulation of HDAC4 and 5. This resulted in decreased proliferation, increased apoptosis and autophagy. Neither adherence to primary BM stromal cells nor growth factors protected against miRNA-9*–dependent growth inhibition.

4.3. miR-9/9* in myeloid malignancies

In AML, miR-9 has been reported to be differentially expressed according to AML subtype. Dependent on the type of leukemic cell, it may suppress or promote leukemic development (**Table 3**, **Figure 5**). The t(8;21) rearrangement is the most common chromosomal translocation in AML and it results in the formation of AML1-ETO fusion protein.[91] AML1-ETO downregulates miR-9 and in this way promotes the expression of UBASH3B/Sts-1, a tyrosine phosphatase that inhibits CBL and enhances STAT5/AKT/ERK/Src signaling to promote myeloid proliferation. Forced expression of miR-9 in t(8;21) AML cells reduces leukemic growth and enhances monocytic differentiation induced by calcitrol by direct repression of the oncogenic LIN28B/HMGA2 axis.[92] LIN28B and HMGA2 are expressed in undifferentiated proliferating cells during embryogenesis and their upregualtion in adult cells leads to oncogenic transformation.

miR-9 is highly upregulated in *MLL*-rearranged leukemic cells as compared to non-*MLL*-rearranged cells and normal controls (**Table 3**, **Figure 5**).[93] MLL fusion proteins may promote miR-9 expression since it is their direct target. Knockdown of endogenous miR-9 expression inhibits MLL fusion–induced immortalization/transformation of normal hematopoietic progenitor cell, whereas its overexpression has the opposite effect. miR-9 function may be mediated by the two predicted targets: RING1 and YY1-binding protein (RYBH) and Ras homolog family member H (RHOH). RYBP is a polycomb complex-associated protein that can stabilize p53 and has tumor suppressor activity. RHOH is a member of the Rho GTPase protein family and it can function as an oncogene or tumor suppressor depending on the context.

In AML patients with normal karyotype, miR-9 is expressed at higher levels in leukemic stem/progenitor cells (LSPCs) than in normal HSC derived from the same patient (**Table 3, Figure 5**).[94] Additionally, miR-9 expression is inversely correlated to the levels of hairy and enhancer of split-1 (HES1), a known tumor-suppressor.[95,96] Knockdown of miR-9 decreases leukemic cell proliferation and survival by direct targeting of HES1 *in vitro* and *in vivo*.[94]

Acute myeloid leukemia

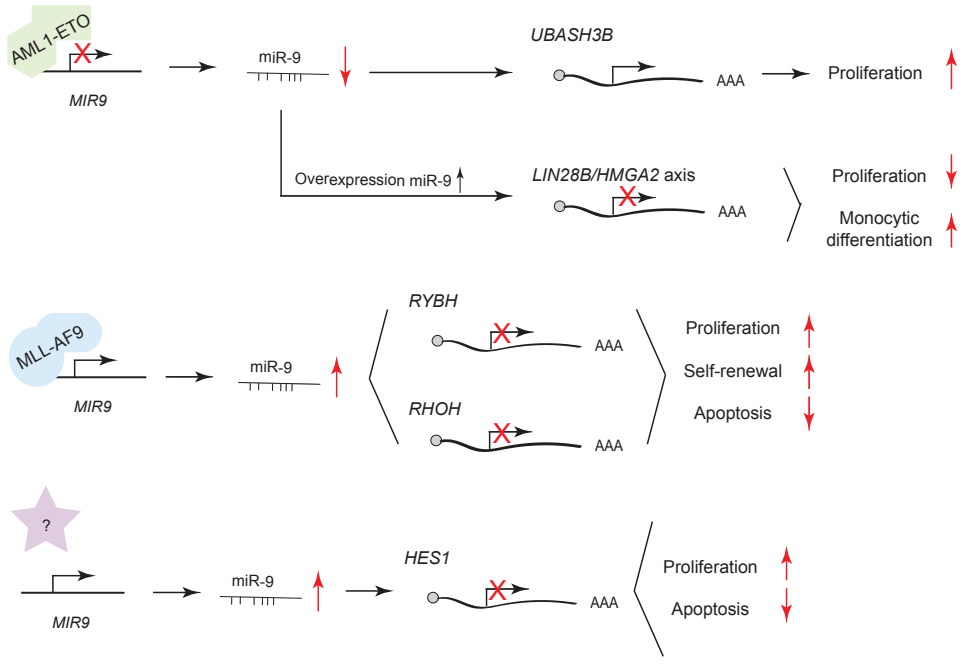

Figure 5. miR-9/9* function in myeloid malignancies. In acute myeloid leukemia (AML), miR-9/9* have been reported to be differentially expressed according to AML subtype, and to suppress or promote leukemic development depending on the cell of context.

SCOPE OF THIS THESIS

miR-9/9* have been shown to be deregulated in different types of human cancer including lymphoid and myeloid malignancies. Nevertheless, we still lack the more comprehensive knowledge about the impact of miR-9/9* expression on normal hematopoietic cell function and their possible impact in the biology of AML.

In **Chapter 2**, we aim to elucidate the function of miR-9/9* in normal myeloid differentiation. We use murine myeloid 32D cell line model and murine primary HSPCs to investigate the impact of miR-9/9* overexpression on granulocytic differentiation induced by G-CSF. We also examine the effect of miR-9/9* expression in human primary AML samples. Further, we set out to identify miR-9/9* targets that are common for murine and human cells and that may be relevant for the observed phenotype. In **Chapter 3**, we broaden our quest for direct and indirect targets that are regulated by miR-9/9* by analyzing proteome changes in 32D cells. We examine the deregulated proteins and the involved pathways that could potentially influence different aspects of normal hematopoietic cell function. Our observations are further extended by *in vivo* and *in vitro* experiments presented in **Chapter 4**, we investigate the influence of miR-9/9* expression on homing of normal HSPCs in the BM.

Until now, few miRNAs have been shown to exhibit a prognostic value in AML. In **Chapter 5**, based on our results about the expression of miR-9/9* in AML patients (shown in **Chapter 1**), we set out to investigate whether the expression of miR-9/9* may predict patient outcome in AML.

Finally, the results presented in this thesis are summarized and discussed in **Chapter 6**. Here we put the accumulated insights about the functions of miR-9/9* into the broader perspective of human cancer.

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2

ABERRANT EXPRESSION OF MIR-9/9* IN MYELOID PROGENITORS INHIBITS NEUTROPHIL DIFFERENTIATION BY POST-TRANSCRIPTIONAL REGULATION OF ERG

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ABSTRACT

Aberrant post-transcriptional regulation by miRNAs has been shown to be involved in the pathogenesis of acute myeloid leukemia (AML). In a previous study, we performed a large functional screen using a retroviral barcoded miRNA expression library. Here, we report that overexpression of miR-9/9* in myeloid 32D cell line (32D-miR-9/9*) had profound impact on GCSF induced differentiation. Further *in vitro* studies showed that enforced expression of miR-9/9* blocked normal neutrophil development in 32D and in primary murine line-agenegative bone marrow cells. We examined the expression of miR-9/9* in a cohort of 647 primary human AMLs. In most cases, miR9 and miR9* were significantly upregulated and their expression levels varied according to AML subtype; with the highest expression in *MLL*-related leukemias harboring 11q23 abnormalities, and the lowest expression in AML cases with t(8;21) and biallelic mutations in *CEBPA*. Gene expression profiling of AMLs with high expression of miR-9/9* and 32D-miR-9/9* identified ETS-related gene (*Erg*) as the only common potential target. Upregulation of ERG in 32D cells rescued miR-9/9*induced block in neutrophil differentiation. Taken together, this study demonstrates that miR-9/9* are aberrantly expressed in most of AML cases and interfere with normal neutrophil differentiation by downregulation of ERG.

INTRODUCTION

Acute myeloid leukemia (AML) is a complex heterogeneous disease characterized by a defect in maturation and the accumulation of immature cells of the myeloid lineage. Many chromosomal and genetic aberrations involved in leukemogenesis have been identified. While the majority of them have functional or prognostic relevance,[1-3] they are insufficient to cause AML alone and other contributing oncogenic events are required for full malignant transformation of hematopoietic precursors.[4-5] In addition to genomic aberrations, changes in epigenetic regulation and post-transcriptional regulation by microRNAs (miRNAs) have been reported.[6-9] The contribution of aberrant miRNA activities to the pathogenesis of AML is still largely unknown.

MiRNAs are short non-coding RNAs (20-25 nt) that bind to specific target sequences within 3'untranslated regions (UTR) of mRNA transcripts.[10-11] This results in transcript cleavage or inhibition of translation, and the subsequent downregulation of expression of their targets.[11-12] MiRNAs have been shown to play an important role in various physiological processes, specific cell fate decisions, and human cancer - including different types of solid tumors[13-14] and leukemia.[15-19] Several studies have shown that cytogenetic subsets of AML have distinct miRNA patterns[7,20-21] and that indeed, some miRNAs, such as miR-223,[15-16] miR-196b,[17] miR-29b,[18] and miR-125b,[19] have established functions in AML.

We and others previously reported about the expression of miR-9 in human AML primary bone marrow cells.[7,20,22-23] MiR-9-5p (miR-9) and miR-9-3p (miR-9*) are two highly conserved miRNAs produced from the same precursor and they are frequently concomitantly expressed.[13,24] Depending on the type of malignancy, miR-9/9* are upregulated (e.g. in glioblastoma[13]) or downregulated (e.g. in breast cancer[14]). Various functions have been assigned to miR-9/9*.[25-30] The function and mode of action of miR-9/9* are cell type-specific. For example, in glioblastoma, downregulation of miR-9/9* promotes neuronal differentiation, suggesting that miR-9/9* influence glioblastoma stem cells and help to maintain their stemness.[13] Conversely, in oral squamous cell carcinoma and non-small cell lung cancer cell lines, overexpression of miR-9 inhibits proliferation and promotes apoptosis.[31-32] MiR-9 activities are cell context-dependent also in AML. Chen *et al.* showed that in MLL-rearranged AML, overexpression of miR-9 enhances MLL-AF9-mediated cell transformation of murine hematopoietic progenitor cells by a yet unknown mechanism.[22] However, Emmrich *et al.* showed that miR-9 has a function in t(8;21) AML and induces monocytic differentiation in KASUMI-1 cell line by targeting the *LIN28B*/*Let-7*/*HMGA2* axis.[23]

Here, we report that enforced expression of miR-9/9* inhibited normal neutrophil development in both myeloid 32D cell line and in primary murine lineage negative bone marrow cells. Additionally, we show that miR-9/9* were significantly upregulated in most cases of primary human AML. Gene expression profiling of AMLs with high expression of miR-9/9* and 32D-miR-9/9* identified ETSrelated gene (*Erg*) as a potential target of miR-9.

Upregulation of ERG in 32D cells rescued miR9/9*induced block in neutrophil differentiation. A block in myeloid differentiation is one of the hallmarks of AML. Hence, our findings uncover a novel function of aberrant expression of miR-9 in myeloid progenitors, which may be relevant for AML development.

MATERIALS AND METHODS

AML and normal bone marrow samples

Patient recruitment and sample processing were performed according to protocols from the Dutch-Belgian-Hematology-Oncology-Cooperative group (HOVON trials 04, 04A, 29, 42, 42A and 43) and the German-Austrian AML Study Group (AMLSG trial HD98A).[7] All trails have been approved by the Institutional Review Board of the Erasmus University Medical Center and the University of Ulm. All patients had a newly diagnosed AML according to the 2001 WHO classification and provided written informed consent in accordance with the Declaration of Helsinki. Mutational analysis was previously described.[33]

Real-time quantitative RT-PCR

Total RNA isolation and real-time quantitative RT-PCR were performed as previously described.[7,34] Briefly, miR-9 and miR-9* expression was determined using real-time quantitative RT-PCR assays for miRNAs (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). Data were normalized using RNU24 with a minimal threshold for Ct values above 35 set to a -dCt value of -15. MiR-9/9* expression in 32D cell line and lineage negative murine bone marrow progenitor cells (Lin- muBM) was determined using a set of endogenous controls, sno202 and sno234, with a minimal threshold for Ct values above 35 set to a -dCt value of -13. The relative quantification method $2^{-(ddCt)}$ was used to calculate the relative expression. Murine *Erg* expression was determined using real-time quantitative RT-PCR assays (Applied Biosystems) with *Hprt* as a normalization control, and human *ERG* using *GAPDH*.

Cell culture and transduction

A retroviral barcoded miRNA library (MSCV-BC-miRNA) was previously reported.[34] Shortly, different miRNAs (plus ~250 bp flanking sequences) were cloned into pMCSV-BC vectors. 32D cells[35] were transduced with MSCV-BC retroviruses and sorted for GFP expression. Different MSCV-BC-miRNA 32D cell populations were mixed with empty vector control infected cells in a 1:1 ratio and cultured in medium containing interleukin 3 (IL-3) or granulocyte colony-stimulating factor (G-CSF). Subsequently, cells were examined as previously described.[34]

The mmu-miR-9-2 precursor (together with ~250 bp flanking sequence) was cloned into a pMSCV retroviral expression system containing GFP.[34] *Erg* cDNA was ordered from the Fantom2 library of Erasmus Center for Biomics (D030036I24; ENSMUST00000077773.7).

Subsequently, *Erg* coding sequence (without the 3' UTR) was cloned into the pLNCX2 retroviral expression system containing a Neomycin resistance gene (Clontech, Mountain View, CA, USA). Retroviral particles were produced as previously described.[34] Lin^- muBM, 32D and NIH3T3 cells were transduced using RetroNectin (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol and selected for GFP expression using FACSAria cell sorter (BD Biosciences, Breda, the Netherlands). Cells overexpressing *Erg* were selected with Neomycin (1mg/ml; Life Technologies, Bleiswijk, the Netherlands).

The 32D cell line was cultured as previously described.[34,35] HEK239T, NIH3T3, and HL-60 cells were cultured according to standard conditions. All cell lines were obtained from DSMZ GmbH (Braunschweig, Germany) and regularly tested for mycoplasma contamination.

MicroRNA LNA inhibition

HL-60 cells were transfected with microRNA LNA non-labeled inhibitors (Exiqon, Vedbaek, Denmark) at the final concentration of 100 nM. FAM-labeled LNA-scramble was used to control for transfection efficiency. At day 5, RNA and protein samples were collected and cell differentiation was evaluated by morphological and flow cytometric analysis. Experiments were performed in three independent replicates.

Methylcellulose colony-forming cell assays

Bone marrow cells were harvested from femurs and tibiae of 8- to 12-week-old C57BL/6 mice (Charles River, Leiden, the Netherlands) and enriched for lineage negative cells using Mouse Hematopoietic Progenitor (Stem) Cell Enrichment Set (BD Biosciences). Colony-forming unit (CFU) cell assays were performed as previously described.[34] Shortly, in CFU assay, 4000 Lin- muBM cells were plated in triplicate in methocult (M3231; StemCell Technologies, Grenoble, France) supplemented with IL-6 (10 ng/mL), IL-3 (supernatant 1/1000), SCF (10 ng/mL) and GM-CSF (10 ng/mL). In CFU-granulocyte (CFU-G) assay, 10000 Lin- muBM cells were plated and cultured with G-CSF (100 ng/mL). For morphological evaluation 100 cells were scored in CFU assay and 200 cells in CFU-G assay. All experiments were performed in three independent biological replicates.

Flow cytometry

Immature hematopoietic progenitor population $Lin^-c\text{-}Kit^+Sca\text{-}1^-$ (LK) was identified by staining with Biotin Mouse Lineage Panel, streptavidin-APC-Cy7, c-Kit-APC (BD Biosciences) and Sca-1-PB (Biolegend, London, United Kingdom). LK population consists of progenitor cells: CMP (common myeloid), GMP (granulocyte-macrophage), and MEP (megakaryocyte-erythroid). For apoptosis analysis, cells were stained with Annexin V:PE Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer's protocol. Data were collected using LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo 7.6.5 software (Tree Star Inc., Ashland, OR, USA).

In microRNA LNA inhibition experiments cells were labeled using EuroFlow protocols with the instrument set-up for neutrophil and monocytic differentiation.[36,37] Data were analyzed using Infinicyt software (Cytognos SL, Salamanca, Spain).

Gene expression profiling analysis

Gene expression profiles (GEP) of 215 AML cases were derived from Human Genome U133 plus 2.0 arrays as previously described (GSE6891).[38] Raw microarray data were processed using MAS5 to target intensity of 100. Intensity values lower than 30 were set to 30 and subsequently log2 transformed. GEP data are available at www.ncbi.nlm.nih.gov/geo (GSE41942). GEP of 32D cells transduced with miR-9/9* (32D-miR-9/9*) or empty vector control (32D-EV) were derived using Mouse Genome 430 2.0 array (Affymetrix, High Wycombe, UK) in three independent experiments. Differentially expressed genes between 32D-miR-9/9* and 32DEV were identified using ANOVA, with the false discovery rate (FDR) calculated using the Benjamini-Hochberg method (FDR<0.05).[38] Only probe sets with standard deviations above 0.05 were included in the analysis using Partek Genomic suite software version 6.4. Potential miR9/9* targets were selected using TargetScan 6.2 www.targetscan.org) web resource.

Western blot

Nuclear extracts were prepared using standard hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl, 10 mM KCl) and high-salt buffer (20 mM HEPES pH 7.9, 25% glycerol, 1.5 mM MgCl, 420 mM NaCl, 0.2 mM EDTA). Specific polyclonal antibodies were used to detect ERG (PA529594; Life Technologies Europe BV, Bleiswijk, the Netherlands). ACTIN (A5441; Sigma-Aldrich, Zwijndrecht, the Netherlands) was used as loading control. Experiments were performed in three or more biological replicates.

3' UTR luciferase reporter assays

The wild-type full length 3' UTR of *ERG* (NM_004449) firefly luciferase reporter construct was obtained from Switchgear Genomics. For site-directed mutagenesis, base pairs 3-6 of the three predicted binding sites targeted by miR-9 were mutated (AAA into GCG) using QuikChange XL Site-Directed Mutagenesis Kit (Agilent, Amstelveen, the Netherlands). HEK293T cells were transfected with firefly reporter constructs together with renilla control plasmids, and MSCV-EV or MSCV-miR-9/9*. Cells were lysed and analyzed with the Dual-Glo Luciferase Reporter Assay (Promega, Madison, WI, USA) according to the manufacturer's protocol. Experiments were performed in three independent biological replicates.

RESULTS

MiR-9/9* inhibits differentiation of early progenitor cells towards neutrophils

In a previous study, we developed a retroviral barcoded miRNA expression library (MSCV-BC-miRNA) and overexpressed more than 130 different miRNAs and empty vector (EV) controls in the 32D cell line model.[34] 32D cells proliferate and maintain their immature myeloblastic morphology in IL-3 containing medium but they are capable to differentiate towards neutrophils within 5 days of G-CSF stimulation.[35] MiR-9/9*overexpressing 32D cells continued to proliferate, whereas control cells and cells overexpressing other miRNAs stopped proliferating after 5 days of G-CSF stimulation (**Figure 1a-b**). This suggests that enforced expression of miR-9/9* interferes with normal neutrophil differentiation.

MiR-9/9* are not expressed in wild type 32D cells (**Supplementary Figure 1a**). To investigate the function of aberrant expression of miR-9/9*, we transduced 32D cells with miR-9/9* (32D-miR-9/9*) or empty vector (32D-EV). Expression of miR-9 and miR-9* was confirmed by qRT-PCR (**Supplementary Figure 1a**). Upon GCSF treatment, 32D-miR-9/9* continued to proliferate as we observed in our initial screen (**Figure 1c**). Additionally, they kept their immature myeloblastic morphology and lost their capacity to differentiate towards neutrophils (**Figure 1d**). To further validate their undifferentiated phenotype, we examined *Cebpa* expression in samples taken at different time points of G-CSF stimulation. Concordant with their immature morphology phenotype, 32D-miR-9/9* cells had lower levels of *Cebpa* than those of 32D-EV cells (**Supplementary Figure 1b**). MiR-9/9* overexpressing 32D cells remained cytokine dependent since withdrawal of IL-3 led to the cell death (data not shown).

To explore the effect of aberrant expression of miR-9/9* in normal early myeloid cells, we performed CFU assays with primary Lin^- muBM. Cells were transduced with MSCV-miR-9/9* or MSCV-EV (**Supplementary Figure 2a**), FACS sorted and plated in methocult. In the CFU assay, after 7 days of culture, miR-9/9* overexpressing cells had a significantly higher frequency of immature hematopoietic progenitor cells $Lin^-c\text{-}Kit^+Sca\text{-}1^-$ cells (LK; miR9/9* – 26%, EV – 12%; P = 0.04; **Figure 2a-b, Supplementary Figure 2c**), and a significantly lower percentage of mature neutrophils (2 fold decrease) based on their morphology (miR-9/9* – 25%, EV – 44%; P = 0.01; **Supplementary Figure 3a-b**). Additionally, miR-9/9* expression did not influence apoptosis (Annexin V^+ population: miR-9/9* – 41%, EV – 35%; P = 0.36; **Supplementary Figure 2d** and **Supplementary Figure 3c**), and no significant differences in colony numbers and size were noted (colony numbers per 1000 cells plated: miR-9/9* – 28, EV – 36; P = 0.11; **Supplementary Figure 3d-e**). A higher frequency of LK population, together with a lower percentage of mature neutrophils and no differences in apoptosis, suggested that miR-9/9* may inhibit granulocytic differentiation. To further address this, we performed CFU-G assays. Again, miR-9/9* overexpressing cells had more than 2 fold less mature neutrophils (miR-9/9* – 11%, EV – 27%; P = 0.04; **Figure 2c-d** and **Supplementary Figure 2b**), and miR-9/9* expression did not influence apoptosis (Annexin V^+ population:

miR-9/9* – 40%, EV – 48%; P = 0.31; **Figure 2e and Supplementary Figure 3f**) and the number of colonies (colony numbers per 1000 cells plated: miR-9/9* – 16, EV – 21; P = 0.31; **Supplementary Figure 3d-e**).

Taken together, this data shows that enforced expression of miR-9/9* inhibits maturation of early progenitor cells towards neutrophils.

MiR-9/9* are aberrantly expressed in AML and their expression is dependent on the AML subtype

We examined miR-9/9* expression in a large cohort of 647 primary human AML cases. In most of AML subtypes, the levels of expression of miR-9/9* were significantly higher than the levels in normal $CD34^+$ BM samples (**Figure 3a-d; Supplementary Table 1 and Supplementary Figure 4a-d**), and the expression of miR-9* correlated highly with miR-9 (r = 0.806). MiR-9/9* levels were the highest in *MLL*-related leukemias harboring 11q23 abnormalities (**Table 1; Figure 3c-d**) and in normal karyotype AML cases with mutations in *NPM1* (**Supplementary Figure 4a-b**). By contrast, AML cases with t(8;21) and bi-allelic mutations in *CEBPA* had the lowest levels of miR9/9*, similar to those in normal BM controls (**Table 1; Figures 3c-d and Supplementary Figure 4a-b**).

***Erg* is a direct miR-9 target that contributes to a block in neutrophil differentiation**

MiRNAs repress their targets by destabilization of mRNA or inhibition of translation.[10-12] To identify miR-9/9* targets that may explain the block in neutrophil differentiation, we analyzed the transcriptomes of AML cells that highly express miR-9/9* and 32D-miR-9/9*.

First, we searched for miR-9/9* targets by the analysis of gene expression data in a cohort of 215 primary AML samples.[38] The cohort was split into two groups based on miR-9/9* levels: the group with the lowest expression (0-25% expression value; lowermost quartile) and with the highest expression (75100% expression value; uppermost quartile). We found 269 probe sets to be significantly differentially expressed between these two groups with an absolute fold change greater than 1.5 (|FC|>1.5). Thirteen probe sets, corresponding to 10 genes, were downregulated in the uppermost miR9/9* quartile and contained predicted miR-9 or miR-9* binding sites according to TargetScan (**Supplementary Table 2**). Next, we performed gene expression profiling of 32D-miR-9/9* cells and 32DEV control cells and found 219 differentially expressed probe sets. Twenty-six probe sets, corresponding to 20 genes, were downregulated in 32D-miR-9/9* cells and had predicted binding sites for miR-9 or miR-9* in their 3' UTRs (**Supplementary Table 3**). A putative miR-9 target ETS-related gene (*Erg*) was the only target that was identified in both sample types, suggesting that it may be functionally involved in miR-9/9*-induced block in neutrophil differentiation (**Figure 4a**). The downregulation of *Erg* transcripts by miR-9 in 32D cells was confirmed by qRT-PCR (**Figure 4b**). All *Erg* isoforms possess all functional domains and have miR-9 binding sites in their 3' UTRs (**Supplementary Figure 5a-d**). In agreement, Western blot analysis in both

murine and human cell lines showed the downregulation of all ERG isoforms upon miR-9/9* overexpression (**Figure 4c, Supplementary Figure 6a**). Additionally, Western blot analysis and gene expression data confirmed the inverse correlation of miR-9/9* and ERG levels in AML patient material (**Supplementary Figure 6b-c**).

To validate *ERG* as a genuine target of miR-9, we performed luciferase reporter assays in HEK293T cells (**Supplementary Figure 7a**). Cells that were transfected with the vector containing the 3' UTR of *ERG* together with the vector containing miR-9/9* had lower luciferase activity than those transfected with the empty vector control (**Supplementary Figure 7b**). The downregulation of luciferase activity by miR-9 was lost when the three putative miR-9 binding sites in 3' UTR of *ERG* were mutated. Endogenous miR-9 is highly expressed in HEK293T cells. Indeed, cells transfected with the wild type 3' UTR of *ERG* had a lower basal luciferase activity, which increased upon mutation of miR-9 binding sites.

To investigate whether *Erg* is involved in the block of neutrophil differentiation in 32D-miR-9/9* cells, we performed rescue experiments using a retroviral pLNCX2 construct containing *Erg* cDNA without 3' UTR. The overexpression was confirmed by Western blot (**Figure 5a**). Upregulation of ERG in 32D-miR-9/9* cells rescued neutrophil differentiation (**Figure 5b-c, Supplementary Figure 7c-d**).

In order to explore the effect of *in vitro* miR-9 knockdown on human AML cell differentiation via modulation of ERG levels, we treated AML cell line HL-60 with microRNA LNA inhibitors. HL-60 cells express endogenous miR-9 (-dCt value of -11) and ERG (-dCt value of -13). All cells were effectively transfected with the inhibitors (data not shown). LNA-miR-9 knockdown resulted in ERG protein upregulation (**Supplementary Figure 8a**). There were no signs of induction of myeloid differentiation according to morphological (**Supplementary Figure 8b**) and flow cytometric analysis of the cells (**Supplementary Figure 8c**).

In conclusion ERG is a validated direct target of miR-9 and it contributes to miR-9/9*-induced block in neutrophil differentiation.

DISCUSSION

AML is a heterogeneous disease that is characterized by a complex interplay of genetic and epigenetic abnormalities. Changes in epigenetic regulation, such as the expression of miRNAs, are known to contribute to the pathogenesis of AML.[15-19] Our *in vitro* studies showed that in 32D and in normal primary Lin$^-$ muBM cells, upregulation of miR-9/9* blocked normal neutrophil development. Recently, two other studies reported on the role of miR-9 in AML. Chen *et al.* showed that overexpression of miR-9 enhanced MLL-AF9-mediated cell transformation in murine hematopoietic progenitor cells *in vitro* and *in vivo*.[22] Mice transplanted with bone marrow progenitors that overexpressed both MLL-AF9 and miR-9 (MLL-AF9+miR-9) had higher frequency of c-Kit+ blast cells in the BM, spleen and peripheral blood than mice transplanted with cells overexpressing MLL-AF9 only. Moreover, morphologically, MLL-AF9+miR-9 leukemic cells had a higher frequency of immature blasts. In line with this, we show that miR-9/9* expression can advocate these changes independent of MLL-AF9. Emmrich *et al.* reported that overexpression of miR-9 induced monocytic differentiation in KASUMI-1 cell line but blocked myelomonocytic differentiation in normal human CD34$^+$ cells.[23] These data, together with our results, indicate that miR-9/9* play a role in myelopoiesis and may be involved in development of AML.

In this study, we report a high expression of miR-9/9* in the most cases of human AML, and their differential expression according to AML subtype; with the highest levels of miR-9/9* in *MLL*-related leukemias harboring 11q23 abnormalities, and the lowest levels in AML cases with t(8;21) and biallelic mutations in *CEBPA*. There was no clear relationship between miR-9/9* expression and different FAB subtypes of AML. Additionally, we previously published that the expression of miR-9/9* is low in distinct differentiation stages of normal human granulopoiesis, with -dCt values similar as in CD34$^+$ cells and ranging from -10 to -15 (data not shown).[39] It is therefore unlikely that these miRNAs are differentially expressed as a result of distinct maturation stages of leukemic cells. As a result, the mechanism of miR-9/9* overexpression in AML is still unknown. In human monocytes and neutrophils, miR-9 was previously reported to be induced upon proinflammatory signals and to be a part of a regulatory feedback loop with NF-κB, a known stress modulator.[29,40-41] One could hypothesize that the upregulation of miR-9/9* in AML may be induced by genetic stress due to gene mutations, deletions or chromosomal aberrations.

MiRNAs regulate various physiological processes and cell fate decisions by downregulating their targets, which may be cell-type-specific. AML is a complex disease that is divided into subtypes, which are characterized by different chromosomal and genetic aberrations. It is likely that miRNAs contribute to the heterogeneity of the disease by downregulating their specific targets. We compared the transcriptomes of cells with high levels of miR-9/9* to transcriptomes of cells with low levels, and we identified several potential targets. ETS-related gene (*Erg*) was the only common gene that was significantly downregulated in both AML and 32D-miR-9/9*. Luciferase reporter assays confirmed that *ERG* was a genuine miR-9 target.

Erg belongs to the ETS family of transcription factors.[42] It is known that *Erg* is essential for definitive hematopoiesis. Several studies in a functionally compromised *Erg* mouse model showed that *Erg* is required for hematopoietic stem cell self-renewal and to maintain normal stem cell function.[43-45] Mice homozygous for a loss-of-function *Erg* allele die at mid-gestation, before the formation of the first hematopoietic stem cells.[43] ERG is known to form homo- and heterodimers as well as ternary complexes with other hematopoietic transcription factors, such as GATA2, RUNX1, FLI-1 and other ETS family proteins.[42,46] Formation of these complexes depends on the availability – relative intracellular concentration – of their components. Therefore, insufficient levels of ERG may influence formation and function of its complexes resulting in aberrant myelopoiesis. On the other side, *Erg* overexpression in a transgenic mouse model revealed promotion of T-acute lymphoblastic leukemia[47] and erythro-megakaryocytic leukemia.[48] Additionally, in a recent study Tursky *et al.* reported that overexpression of *ERG* in normal human CD34$^+$ cord blood cells enhanced expansion of the progenitor pool but did not interfere with myeloid differentiation.[49] Thus, ERG plays an important role in normal and malignant hematopoiesis, and ERG function depends on its level of expression.

Here, we show that ERG is downregulated by miR-9, and that the upregulation of ERG reverses miR-9/9*-induced block in normal neutrophil differentiation. This suggests that downregulation of ERG by miR9 interferes with normal myelopoiesis. In human AML cell line HL-60, LNA-based miR-9 knockdown led to upregulation of ERG protein levels but was insufficient to revert the block in differentiation phenotype in this malignant transformed *in vitro* model.

In conclusion, our findings uncover a novel function of aberrant miR-9 expression in myeloid differentiation. However, its role in leukemogenesis remains unsettled.

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Authorship

Contribution: K.N. and S.M.S. contributed equally to the manuscript. K.N. and S.M.S. planned, carried out the experiments and analyzed the data. L.B. and H.D. provided the German dataset and carried out experiments. K.vL. performed morphological evaluation and quantification of cytospins. S.J.E., C.E., and M.K.D. performed experiments. E.M.J.B., S.J.E., H.D., L.B., B.L. and M.J.L. designed the study and interpreted the results. K.N., S.M.S., S.J.E., L.B., H.D., B.L. and M.J.L. wrote or contributed to the manuscript.

Table 1. AML cohort characteristics and relationships with miR-9/9* expression

	Number of cases	miR-9		miR-9*	
		P	Median difference/Rho	P	Median difference/Rho
Clinical parameters					
Age (mean)	45.4	0.044	rho=0.0792	0.037	rho=0.0821
Range (min-max)	15.0-77.0				
WBC, x 10 9 /L (mean)	47.4	0.038	rho=0.0819	0.091	rho=0.0666
Range (min-max)	0.3-510.0				
Sex					
Male	335	0.025	-0.50	0.126	-0.34
Female	312	0.025	0.50	0.089	0.38
ELN genetic risk (modified)					
Favorable	234	<0.001	-1.72	<0.001	-1.13
Intermediate-I	194	<0.001	0.98	<0.001	0.95
Intermediate-II	119	0.021	0.61	0.165	0.39
Adverse	102	0.372	0.38	0.870	0.02
Cytogenetics					
+8	33	0.163	0.05	0.095	0.79
-5 or -5q	3				
-7 or -7q	19	0.006	-1.55	0.017	-1.49
-9q	13	0.012	-2.14	0.102	-1.28
11q23†	20	<0.001	2.42	0.048	1.45
t(8;21)	39	<0.001	-3.38	<0.001	-2.77
t(9;11)	15	<0.001	3.96	<0.001	3.94
t(15;17)	28	0.001	-1.66	0.007	-1.43
inv(3) or t(3;3)	7				
inv(16)	48	0.009	-0.74	0.022	-0.82
Normal karyotype	307	<0.001	0.80	<0.001	0.95
Complex karyotype	61	0.986	0.00	0.326	-0.38
Other	71	0.192	0.46	0.948	-8.46
Molecular genetics‡					
CEBPA single mutant	11	0.370	-0.57	0.824	0.29
CEBPA double mutant	34	<0.001	-3.64	<0.001	-2.46
FLT3-ITD	117	<0.001	1.07	<0.001	1.04
FLT3-TKD	39	<0.001	1.75	0.003	1.37
NPM1	166	<0.001	<0.001	<0.001	1.51

Table reports P-values of Spearman correlation test (together with Spearman correlation coefficients; positive rho coefficient indicates a positive relationship, negative/positive rho indicates a negative/positive relationship between the two continuous variables) or Wilcoxon rank sum test (together with median differences; a negative/positive median difference indicates lower/higher miR-9 or miR-9* in the specific subtype bearing the abnormality). Spearman correlation test or Wilcoxon rank sum test were used to assess the association between continuous miR-9 or miR-9* expression and different clinical and molecular parameters with more than 10 cases.
In ELN favorable risk group only patients with biallelic mutations in *CEBPA* were included.
†11q23 category contains *MLL*-related leukemias harboring 11q23 abnormalities other than t(9;11).
‡Molecular data only determined in patients with normal karyotype.
Abbreviations: AML, acute myeloid leukemia; WBC, white blood cell count; ELN, European LeukemiaNet; CEBPA, CCAAT/enhancer-binding protein α; FLT3-ITD, internal tandem duplications in the FMS-like tyrosine kinase 3; FLT3-TKD, FMS-like tyrosine kinase 3 with mutations in tyrosine kinase domain; NPM1, nucleophosmin.

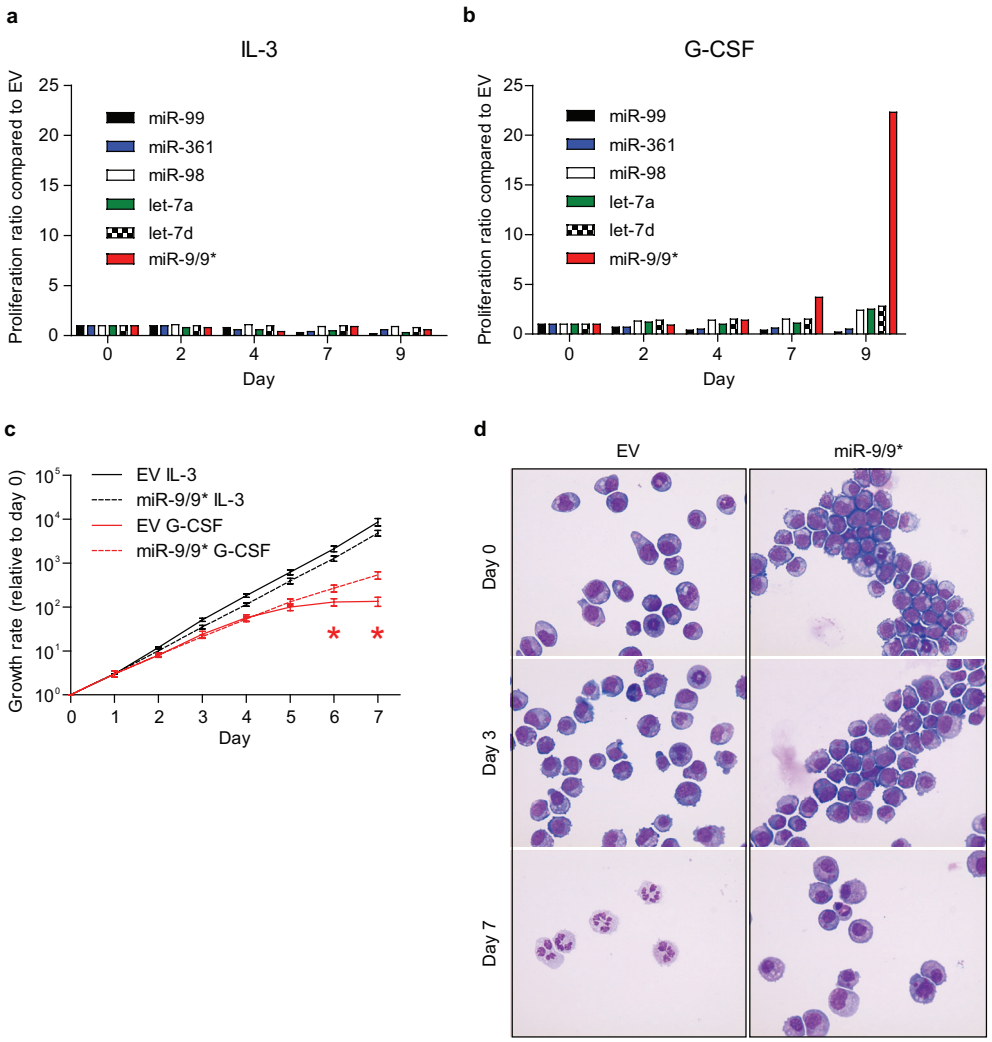

Figure 1. MiR9/9* inhibits granulocytic maturation in murine myeloid 32D cell line. 32D cells were transduced with a retroviral barcoded miRNA library (MSCVBCmiRNA), randomly assigned to separate pools and differentiated upon GCSF treatment. Proliferation ratios of cells cultured upon (**a**) IL3 or (**b**) GCSF in the same pool as cells overexpressing miR9/9* are given relatively to three empty vector (EV) controls. Data show one biological replicate. To investigate the phenotype of aberrant upregulation of miR9/9*, 32D cells were separately transduced with MSCVEV (EV) or MSCVmiR9/9* (miR9/9*) retroviral construct, FACS sorted, and differentiated upon G-CSF stimulation. (**c**) Average growth rates of cells transduced with EV control (solid line) or overexpressing miR9/9* (dashed line); upon IL3 (black) or GCSF (red) stimulation. Growth rates are given as number of cells at each time point divided by number of cells at day 0. Data show an average of five independent biological replicates. Error bars represent the standard deviation. Statistically significant P-values between EV and miR-9/9* transduced cells upon G-CSF treatment are marked as red stars. (**d**) Representative micrographs of cytospins stained with May Grünwald Giemsa (original magnification ×100). Unpaired twotailed t-test was used for the statistical analysis. *P < 0.05.

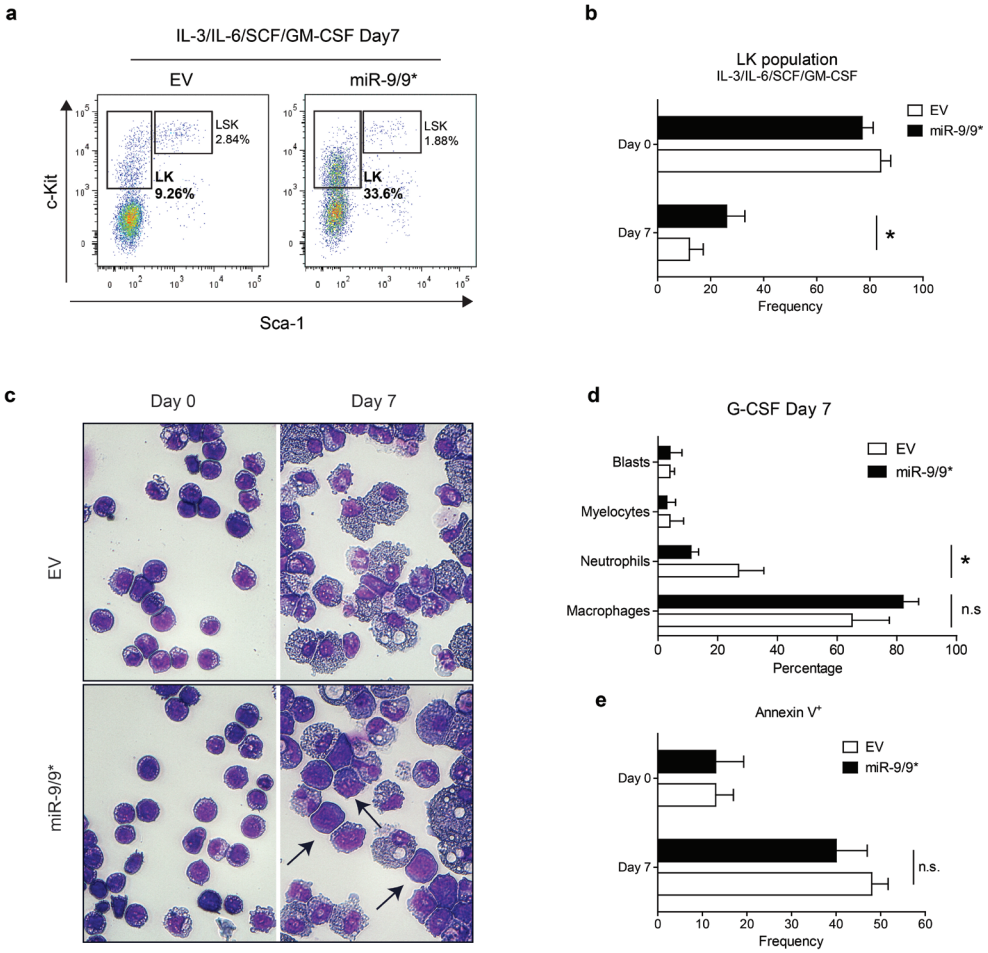

Figure 2. MiR9/9* inhibits maturation of Lin⁻ muBM cells towards neutrophils. Lin⁻ muBM cells were transduced with MSCVEV (EV) or MSCVmiR9/9* (miR9/9*) retroviral construct, FACS sorted, and plated in triplicate in methocult. (**a**) Representative FACS plot showing Lin⁻c-Kit⁺Sca-1⁻ (LK) progenitor populations in one replicate at day 7 of CFU assay. (**b**) A summary of LK populations in all three replicates at day 0 and day 7 of CFU assay. (**c**) Representative micrographs of cytospins stained with May Grünwald Giemsa at day 0 and day 7 of CFUG assay (original magnification ×100). Black arrows highlight immature miR-9/9* cells. (**d**) A summary of distinct differentiation stages scored based on morphology at day 7 of CFUG assay. (**e**) A summary of Annexin V⁺ populations in all three replicates at day 0 and day 7 of CFUG assay. All experiments were performed in three independent biological replicates. Unpaired twotailed t-test was used for the statistical analysis. *P < 0.05.

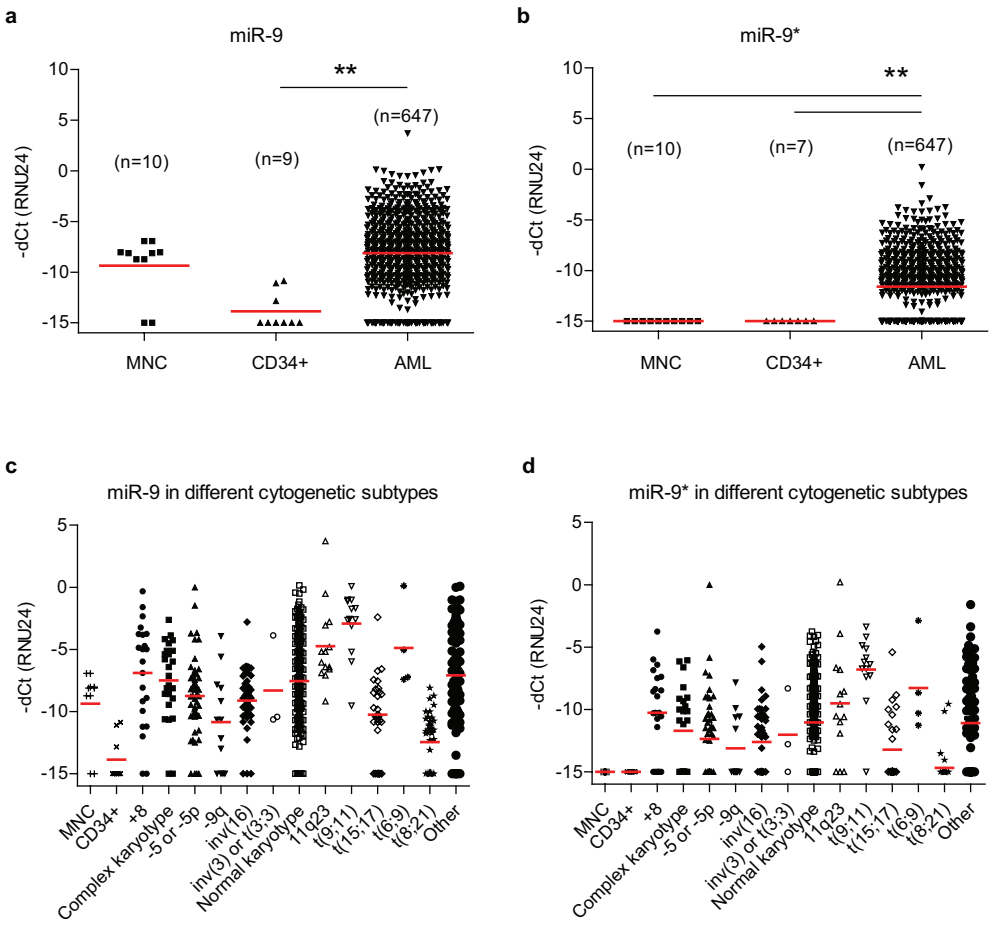

Figure 3. MiR9/9* are aberrantly expressed in AML and their expression is dependent on the AML subtype. Quantitative RTPCR of miR9 and miR9* in a cohort of primary human AML cases, normal human mononuclear cells (MNC), and myeloblast cells ($CD34^+$) isolated from bone marrow. (**a**) MiR9 and (**b**) miR9* expression in all AML cases, and (**c-d**) in different cytogenetic subtypes of AML. The 11q23 category contains *MLL*-related leukemias harboring 11q23 abnormalities other than t(9;11). RNU24 was used as endogenous control. Expression is given as dCt, where higher values represent higher expression. For measurements below detection, the minimal threshold was set to dCt value of 15. Unpaired two-tailed t-test was used for the statistical analysis. **P < 0.001. For detailed information about the miR-9/9* expression levels in different subtypes see **Supplementary Table 1.**

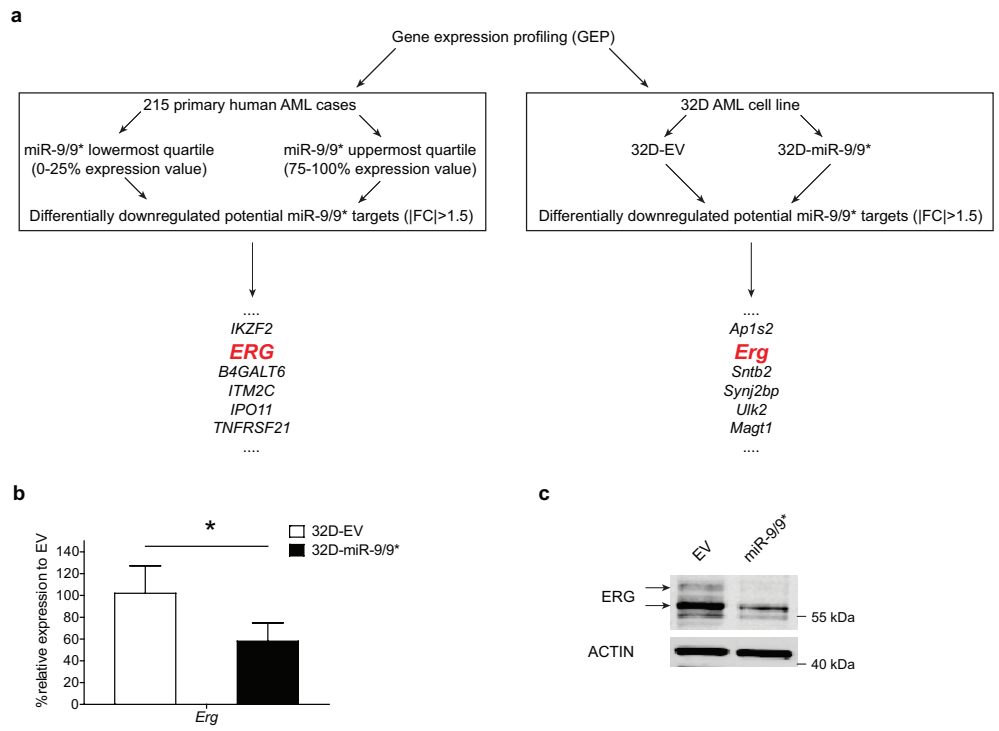

Figure 4. MiR9/9* target identification. (**a**) A summary of miR9/9* target identification by transcriptome analysis. ETS-related gene (*Erg*) was the only target identified in both sample types. (**b**) Quantitative RTPCR and (**c**) Western blot analysis of ERG in 32DmiR-9/9*. All experiments were performed in three independent biological replicates. Unpaired two-tailed t-test was used for the statistical analysis. *P < 0.05.

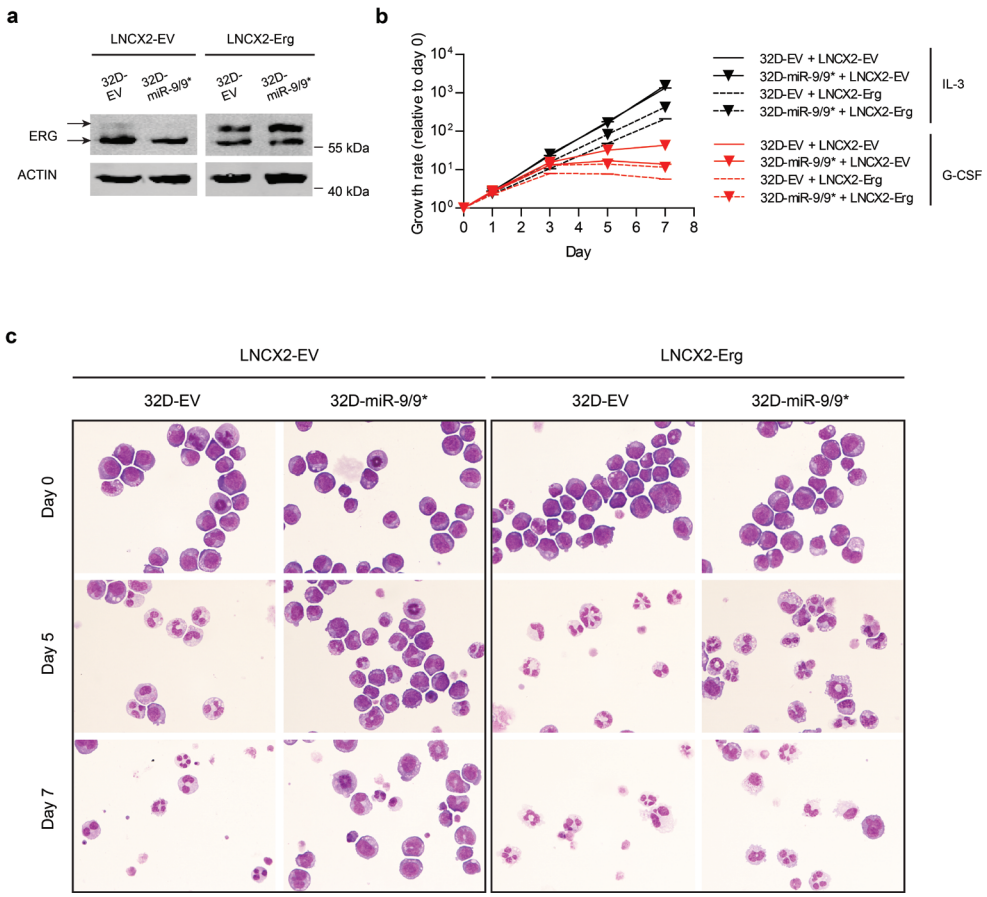

Figure 5. ERG upregulation reverses block in neutrophil differentiation induced by miR9/9* overexpression. (**a**) Western blot analysis of 32DEV and 32DmiR9/9* cells transduced with LNCX2EV or LNCX2Erg. (**b**) Average growth rates of 32DEV (no symbol) and 32DmiR9/9* (triangle) cells that were transduced with LNCX2EV (solid line) or LNCX2Erg (dashed line); cultured upon IL3 (black) or GCSF (red) stimulation. Growth rates are given as number of cells at each time point divided by number of cells at day 0. Data show one representative biological replicate. (**c**) Representative micrographs of cytospins stained with May Grünwald Giemsa at different time points of GCSF induced differentiation (original magnification ×100). All experiments were performed in two independent biological replicates.

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SUPPLEMENTARY DATA

Table S1. MiR-9/9* expression level in different AML subtypes. RNU24 was used as endogenous control. Expression is given as -dCt, where higher values represent higher expression. For measurements below detection, the minimal threshold was set to -dCt value of -15. Unpaired two-tailed t-test was used for the statistical analysis.

Variable	Mean	Std. Dev.	P (MNC)*	P (CD34⁺)*
miR-9				
MNC	-9.363	3.033		
CD34⁺	-13.870	1.779		
AML	-8.122	3.748	0.298	<0.001
+8	-6.902	4.177	0.106	<0.001
Complex karyotype	-7.494	3.195	0.122	<0.001
-5 or -5q	-8.755	3.192	0.581	<0.001
-9q	-10.852	3.768	0.320	0.022
inv(16)	-9.114	2.224	0.761	<0.001
inv(3) or t(3;3)	-8.314	3.847	0.628	0.121
Normal karyotype	-7.542	3.555	0.110	<0.001
11q23	-4.747	3.245	0.002	<0.001
t(9;11)	-2.927	2.599	<0.001	<0.001
t(15;17)	-10.253	3.111	0.440	<0.001
t(6;9)	-4.888	3.510	0.034	0.010
t(8;21)	-12.455	2.212	0.001	0.058
Other	-7.097	4.021	0.088	<0.001
NPM1 wt	-8.905	4.193	0.736	<0.001
NPM1 mutant	-6.449	2.530	<0.001	<0.001
FLT3 wt	-8.388	3.924	0.442	<0.001
FLT3-ITD	-6.944	3.024	0.017	<0.001
FLT3-TKD	-5.898	2.454	<0.001	<0.001
CEBPA wt	-6.787	3.022	0.009	<0.001
CEBPA mutant	-11.653	3.562	0.064	0.010
CEBPA double mutant	-12.457	3.435	0.014	0.103
CEBPA single mutant	-8.948	2.820	0.749	<0.001
miR-9*				
MNC	-15.000	0.000		
CD34⁺	-15.000	0.000		
AML	-11.598	3.294	<0.001	<0.001
+8	-10.262	3.456	<0.001	<0.001
Complex karyotype	-11.708	3.240	<0.001	<0.001
-5 or -5q	-12.372	3.210	<0.001	<0.001
-9q	-13.115	2.609	0.023	0.023
inv(16)	-12.615	2.618	<0.001	<0.001
inv(3) or t(3;3)	-12.032	3.404	0.270	0.270
Normal karyotype	-11.045	3.161	<0.001	<0.001
11q23	-9.515	4.325	<0.001	<0.001
t(9;11)	-6.803	2.897	<0.001	<0.001
t(15;17)	-13.233	2.672	0.020	0.020
t(6;9)	-8.286	3.759	0.038	0.038
t(8;21)	-14.697	1.129	0.090	0.090
Other	-11.106	3.663	<0.001	<0.001
NPM1 wt	-11.913	3.430	<0.001	<0.001
NPM1 mutant	-10.341	2.741	<0.001	<0.001
FLT3 wt	-11.563	3.312	<0.001	0.004
FLT3-ITD	-10.659	2.908	<0.001	<0.001
FLT3-TKD	-10.157	2.851	<0.001	<0.001
CEBPA wt	-10.577	3.029	<0.001	<0.001
CEBPA mutant	-13.617	2.566	<0.001	<0.001
CEBPA double mutant	-14.257	2.112	0.002	0.002
CEBPA single mutant	-11.386	2.882	0.048	0.048

Expression level is given as -dCt. RNU24 was used as endogenous control.
*P-value of unpaired two-tailed t-test. Each variable was compared to MNC or CD34⁺ cells.
Abbreviations: AML, acute myeloid leukemia; MNC, mononuclear cells; WBC, white blood cell count; CEBPA, CCAAT/enhancer-binding protein α; NPM1, nucleophosmin; wt, wild type; FLT3-ITD, internal tandem duplications in the FMS-like tyrosine kinase 3; FLT3-TKD, FMS-like tyrosine kinase 3 with mutations in tyrosine kinase domain.
See also **Table 1**, **Figure 3**, and **Supplementary Figure 4**.

Table S2. Significantly downregulated probe sets in AML patients with high miR-9/9* expression. 0-25% expression value (lowermost quartile) vs 75-100% expression value (uppermost quartile) with Padjusted < 0.05 and fold change > 1.5.

		0-25% vs 75-100%		Target prediction algorithm	
Probe set ID	Gene symbol	Padjusted	Fold change	TargetScan (miR-9)	TargetScan (miR-9*)
214953_s_at	APP	3.24E-08	3.64		
206622_at	TRH	2.22E-07	3.55		
200602_at	APP	6.96E-08	3.32		
202746_at	ITM2A	7.87E-08	3.11		
201015_s_at	**JUP***	1.30E-11	3.07	x	
206726_at	PGDS	3.47E-08	3.04		
218825_at	EGFL7	1.09E-11	3.00		
217979_at	TSPAN13	3.40E-10	2.99		
241133_at	---	1.15E-06	2.96		
219837_s_at	CYTL1	3.13E-07	2.94		
206660_at	IGLL1	5.98E-06	2.89		
225285_at	BCAT1	1.89E-07	2.85		
223708_at	C1QTNF4	3.34E-05	2.77		
202747_s_at	ITM2A	1.79E-06	2.73		
206761_at	CD96	1.94E-06	2.70		
227923_at	SHANK3	1.40E-06	2.69		
200665_s_at	SPARC	4.49E-06	2.68		
202947_s_at	GYPC	2.99E-09	2.67		
231929_at	**IKZF2**	8.45E-08	2.66	x	x
213541_s_at	**ERG**	1.52E-11	2.65	x	
202242_at	TSPAN7	4.47E-08	2.59		
202016_at	MEST	8.20E-06	2.58		
235333_at	**B4GALT6**	5.43E-09	2.56		x
209583_s_at	CD200	3.31E-06	2.53		
231982_at	LOC284422	6.87E-07	2.52		
207550_at	MPL	1.64E-08	2.48		
235142_at	LOC730411 /// ZBTB8	3.53E-08	2.48		
208116_s_at	MAN1A1	3.14E-09	2.43		
226517_at	BCAT1	6.62E-07	2.41		
229002_at	FAM69B	6.08E-09	2.40		
237849_at	---	2.81E-08	2.35		
221760_at	MAN1A1	1.59E-08	2.32		
227230_s_at	KIAA1211	3.76E-08	2.32		
224428_s_at	CDCA7	7.63E-09	2.30		
206233_at	**B4GALT6**	1.49E-08	2.29		x
213258_at	TFPI	9.12E-06	2.28		
221004_s_at	**ITM2C**	5.11E-07	2.25	x	
209160_at	AKR1C3	2.79E-06	2.24		
238488_at	**IPO11**	1.65E-11	2.23	x	
209771_x_at	CD24	5.26E-04	2.19		
241926_s_at	**ERG**	9.55E-11	2.16	x	
221942_s_at	GUCY1A3	1.25E-04	2.15		
216379_x_at	CD24	1.68E-04	2.13		
227235_at	---	2.26E-04	2.10		
204960_at	PTPRCAP	8.87E-08	2.07		
207134_x_at	TPSAB1	1.80E-02	2.07		
217023_x_at	TPSAB1	8.99E-03	2.07		
205683_x_at	TPSAB1	2.37E-02	2.06		
205051_s_at	KIT	8.89E-04	2.05		
266_s_at	CD24	2.14E-04	2.04		
210084_x_at	TPSAB1	2.43E-02	2.04		
215382_x_at	TPSAB1	2.36E-02	2.04		
216733_s_at	GATM	3.19E-05	2.03		
218856_at	**TNFRSF21**	1.69E-05	2.02	x	
216474_x_at	TPSAB1	1.79E-02	2.01		
208651_x_at	CD24	5.56E-04	2.00		
203178_at	GATM	1.86E-04	1.97		
204165_at	WASF1	1.69E-04	1.97		
229530_at	---	3.76E-04	1.97		
232227_at	---	1.74E-09	1.97		
218858_at	DEPDC6	1.87E-05	1.93		
235171_at	---	3.83E-04	1.90		
209795_at	CD69	2.59E-03	1.89		
220059_at	BRDG1	1.97E-05	1.88		
227860_at	CPXM1	7.51E-04	1.88		
205240_at	GPSM2	1.02E-06	1.87		
212558_at	SPRY1	3.20E-04	1.87		x
200953_s_at	**CCND2**	3.15E-06	1.86		
208650_s_at	CD24	1.38E-03	1.85		
203608_at	ALDH5A1	1.50E-04	1.85		
226473_at	CBX2	2.53E-06	1.85		
214452_at	BCAT1	7.60E-07	1.84		
218966_at	MYO5C	7.56E-08	1.83		
224759_s_at	C12orf23	8.50E-06	1.82		
228029_at	ZNF721	1.89E-05	1.82		
209560_s_at	DLK1	1.84E-03	1.80		
219686_at	STK32B	2.01E-06	1.80		
213668_s_at	**SOX4**	1.62E-02	1.79		x
203069_at	SV2A	1.60E-04	1.78		
227231_at	KIAA1211	2.12E-06	1.78		
1555120_at	CD96	5.33E-07	1.77		
201416_at	**SOX4**	6.37E-03	1.77		x
229801_at	C10orf47	1.28E-08	1.76		
219271_at	GALNT14	1.44E-04	1.75		
214390_s_at	BCAT1	3.87E-05	1.73		
226043_at	GPSM1	2.14E-04	1.73		
229390_at	FAM26F	9.51E-03	1.70		
201839_s_at	TACSTD1	4.25E-03	1.69		
210140_at	CST7	2.27E-02	1.69		
204529_s_at	TOX	1.62E-02	1.66		
201830_s_at	NET1	1.20E-03	1.64		
223075_s_at	**C9orf58**	1.61E-02	1.61		x
210933_s_at	FSCN1	1.65E-02	1.56		
201564_s_at	FSCN1	1.11E-02	1.55		
225105_at	OCC-1	3.60E-03	1.51		
223565_at	MGC29506	3.28E-02	1.50		

x: Potenti*l target of miR-9 or miR-9* that was predicted by TargetScan algorithm.
: Potential targets of miR-9 or miR-9 that were downregulated with a fold change>1.5 are marked in red.

Table S3. Significantly downregulated probe sets in 32D-miR-9/9*. Significantly downregulated probe sets in 32D-miR-9/9* cells compared to those in 32D-EV cells with Padjusted < 0.05 and fold change > 1.5.

				Target prediction algorithm	
Probe set ID	Gene symbol	Padjusted	Fold change	TargetScan (miR-9)	TargetScan (miR-9*)
1420394_s_at	Gp49a /// Lilrb4	8.98E-03	3.26		
1448301_s_at	Serpinb1a	7.19E-03	2.95		
1428478_at	**Reep4***	4.63E-06	2.73	x	
1434940_x_at	Rgs19	7.50E-05	2.55		
1417588_at	**Galnt3**	3.16E-08	2.40	x	
1416318_at	Serpinb1a	4.02E-02	2.39		
1425469_a_at	---	5.22E-03	2.22		
1425764_a_at	**Bcat2**	8.12E-05	2.15	x	
1419091_a_at	**Anxa2**	8.43E-05	2.10	x	
1425471_x_at	---	4.10E-02	2.09		
1436522_at	**Map3k3**	2.14E-06	2.08	x	
1419648_at	**Myo1c**	1.23E-04	2.06	x	
1454942_at	Fam129a	5.20E-04	2.02		
1418448_at	Rras	4.79E-04	2.00		
1422861_s_at	Pdlim5	2.58E-03	1.98		
1420172_at	---	2.89E-03	1.96		
1456179_at	AU019176	2.68E-02	1.94		
1429364_at	4930579G24Rik	5.36E-04	1.94		
1437232_at	Bpil2	1.22E-02	1.94		
1451475_at	Plxnd1	7.94E-04	1.91		
1456150_at	**Jhdm1d**	2.16E-03	1.90		x
1422567_at	Fam129a	2.36E-03	1.89		
1420827_a_at	**Ccng1**	1.42E-03	1.87	x	
1416472_at	**Syap1**	4.06E-03	1.85	x	
1449020_at	Plscr3	6.20E-03	1.82		
1420170_at	**Myh9**	3.41E-03	1.82	x	
1423156_at	**Gnpnat1**	1.16E-02	1.79	x	
1425122_at	Fam3b	1.64E-03	1.77		
1418468_at	100039484 /// 100039503 /// Anxa11	5.25E-04	1.75		
1456057_x_at	**Tmem109**	9.15E-05	1.74	x	
1437008_x_at	**Tmem109**	8.00E-04	1.74	x	
1420725_at	Tmlhe	7.05E-03	1.73		
1444015_at	---	1.70E-03	1.73		
1460036_at	**Ap1s2**	4.56E-03	1.73		x
1452254_at	Mtmr9	5.64E-04	1.73		
1440244_at	**Erg**	4.08E-02	1.72	x	
1417472_at	**Myh9**	4.36E-03	1.72	x	
1419455_at	Il10rb	2.79E-03	1.70		
1453299_a_at	LOC100045567 /// Pnp1 /// Pnp2	1.15E-03	1.70		
1440388_at	---	1.73E-03	1.70		
1419649_s_at	**Myo1c**	7.57E-06	1.70	x	
1420372_at	**Sntb2**	8.14E-04	1.70		x
1417786_a_at	Rgs19	7.26E-04	1.69		
1423707_at	Tmem50b	7.25E-03	1.68		
1449180_at	Kcmf1	6.38E-04	1.67		
1427918_a_at	Rhoq	6.49E-03	1.66		
1452655_at	Zdhhc2	1.44E-03	1.66		
1416032_at	**Tmem109**	2.74E-02	1.65	x	
1439959_at	Fgf11	4.53E-03	1.63		
1423614_at	Lrrc8c	2.58E-03	1.62		
1417471_s_at	D1Ertd622e	4.73E-03	1.62		
1423295_at	Tm9sf2	1.99E-02	1.62		
1417834_at	**Synj2bp**	6.18E-03	1.61	x	
1450786_x_at	Pdlim5	6.43E-03	1.61		
1438396_at	Ocrl	8.53E-03	1.60		
1424834_s_at	Itpr2	1.03E-02	1.60		
1431072_a_at	Ccdc50	7.33E-04	1.60		
1435867_at	**Jhdm1d**	2.87E-02	1.59	x	
1457281_at	Dnajc21	9.80E-03	1.58		
1417847_at	**Ulk2**	8.19E-03	1.58	x	
1427475_a_at	Pdlim5	7.10E-04	1.58		
1420382_at	Apob48r	3.41E-02	1.58		
1449383_at	Adssl1	1.68E-03	1.57		
1436213_a_at	1110028C15Rik	4.36E-02	1.57		

x: Potential target of miR-9 or miR-9* that was predicted by TargetScan algorithm.
: Potential targets of miR-9 or miR-9 that were downregulated with a fold change>1.5 are marked in red.

Probe set ID	Gene symbol	Padjusted	Fold change	Target prediction algorithm	
				TargetScan (miR-9)	TargetScan (miR-9*)
1435777_at	Itpripl2	1.33E-03	1.57		
1441906_x_at	**Syap1**	7.53E-03	1.56	x	
1448853_at	Synj2bp	6.73E-05	1.56		
1419459_a_at	**Magt1**	1.36E-03	1.55	x	
1428622_at	Depdc6	3.78E-02	1.55		
1428633_at	Twistnb	2.44E-02	1.54		
1417398_at	Rras2	5.87E-03	1.54		
1437830_x_at	Zbed3	1.94E-02	1.53		
1438932_at	Rasgrp2	4.76E-02	1.53		
1452013_at	Atp10a	7.69E-03	1.53		
1419029_at	Ero1l	4.35E-02	1.53		
1444426_at	Cass4	2.15E-02	1.52		
1433571_at	Serinc5	4.58E-02	1.52		
1420171_s_at	**Myh9**	5.72E-03	1.52	x	
1450980_at	Gtpbp3	4.55E-02	1.52		
1457202_at	---	3.18E-02	1.51		
1424746_at	Kif1c	7.27E-03	1.51	x	
1455787_x_at	Minpp1	1.98E-02	1.50		
1452521_a_at	Plaur	1.96E-02	1.50		

x: Potential target of miR-9 or miR-9* that was predicted by TargetScan algorithm.
: Potential targets of miR-9 or miR-9 that were downregulated with a fold change>1.5 are marked in red.

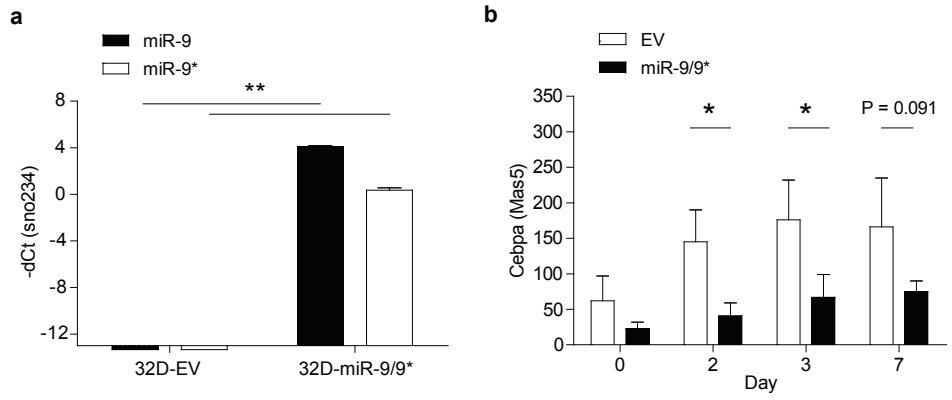

Figure S1. MiR-9/9* inhibits granulocytic maturation in murine myeloid 32D cell line. (**a**) Overexpression of miR9/9* showed by qRT-PCR in cells that were transduced with empty vector (32D-EV) or with vector containing miR-9/9* precursor (32D-miR-9/9*). Sno234 was used as endogenous control. Expression is shown as -dCt, where higher values represent higher expression. (**b**) *Cebpa* expression in samples taken at different time points of G-CSF induced differentiation measured with Mouse Genome 430 2.0 array. All experiments were performed in three independent biological replicates. Unpaired two-tailed t-test was used for the statistical analysis. *P < 0.05. **P < 0.001.

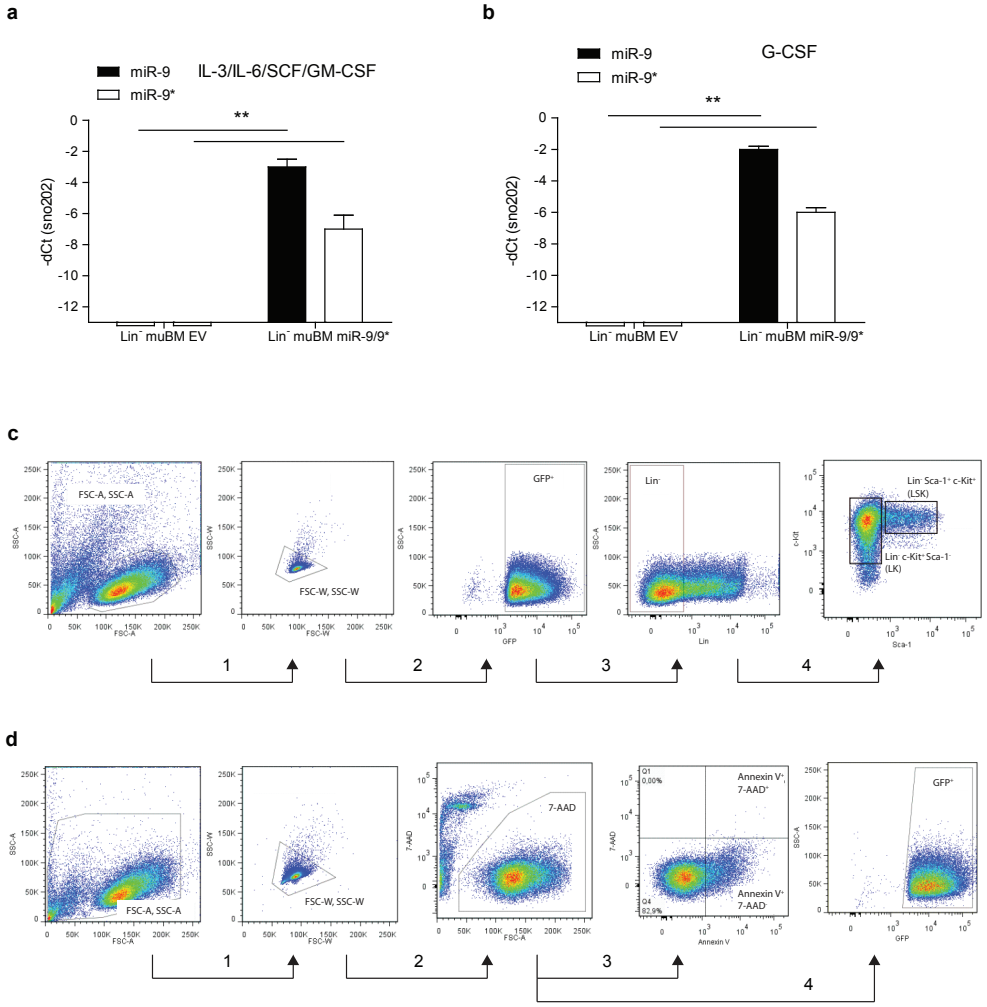

Figure S2. MiR-9/9* overexpression and FACS gating strategy in colony-forming cell assays. (**a-b**) qRT-PCR in lineage negative murine bone marrow cells (Lin⁻ muBM) transduced with empty vector (EV) or with vector containing miR-9/9* precursor (miR-9/9*), in (**a**) CFU and (**b**) CFU-G assay. Sno202 was used as endogenous control. Expression is shown as-dCt, where higher values represent higher expression. (**c**) FACS gating strategy for defining Lin⁻c-Kit⁺Sca-1⁻ (LK) progenitor population. One biological replicate is shown at day 0 of CFU assay. (**d**) FACS gating strategy for defining Annexin V⁺ population. One biological replicate is shown at day 0 of CFU-G assay. Unpaired two-tailed t-test was used for the statistical analysis. **P < 0.001.

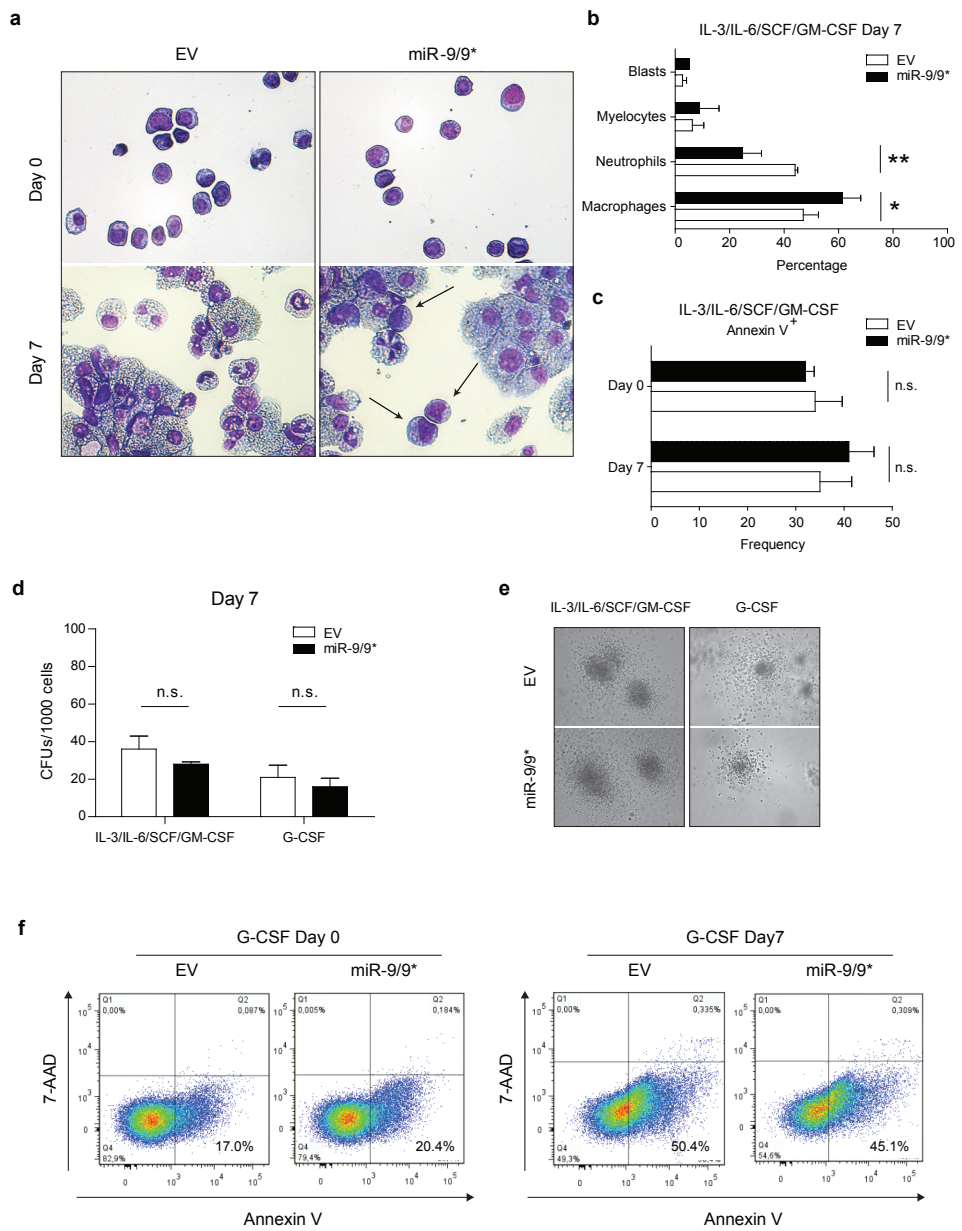

Figure S3. Cell morphology, apoptosis and colony characteristic in colony-forming cell assays. (**a**) Representative micrographs of cytospins stained with May Grünwald Giemsa (original magnification ×100). Black arrows highlight immature miR-9/9* cells. (**b**) A summary of distinct differentiation stages scored based on morphology at day 7 of CFU assay. (**c**) A summary of Annexin V⁺ populations at day 0 and day 7 of CFU assay. (**d**) A summary of colony numbers (CFUs) per 1000 cells plated at day 7 of CFU and CFU-G assays. (**e**) Representative micrographs of colonies at day 7 of CFU and CFU-G assays (original magnification ×40). (**f**) Representative FACS plots showing Annexin V⁺ populations in one replicate at day 0 and day 7 of CFU-G assay. All experiments were performed in three indendent biological replicates. Unpaired two-tailed t-test was used for the statistical analysis. *P < 0.05. **P < 0.001.

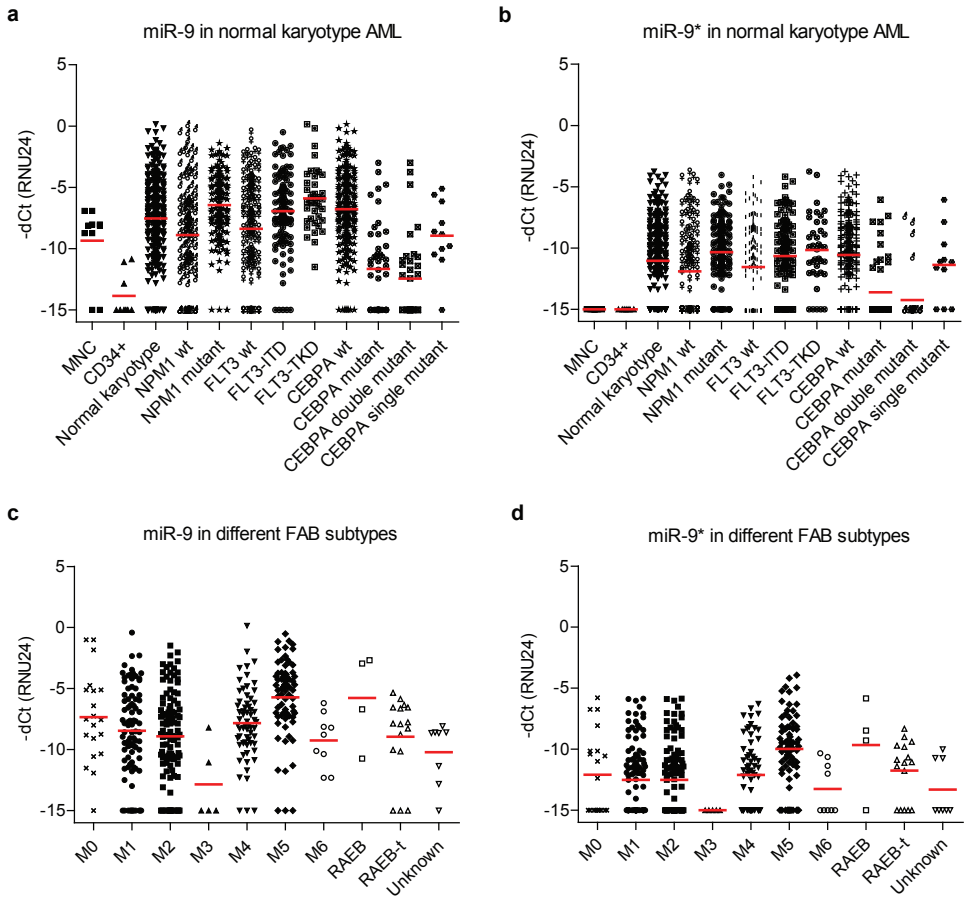

Figure S4. MiR-9/9* expression in AML. (**a**) MiR-9 and (**b**) miR-9* expression in different subtypes of normal karyotype AML. (**c**) MiR-9 and (**d**) miR-9* expression in different FAB subtypes. RNU24 was used as endogenous control. Expression is given as-dCt. For measurements below detection, the minimal threshold was set to-dCt value of-15.

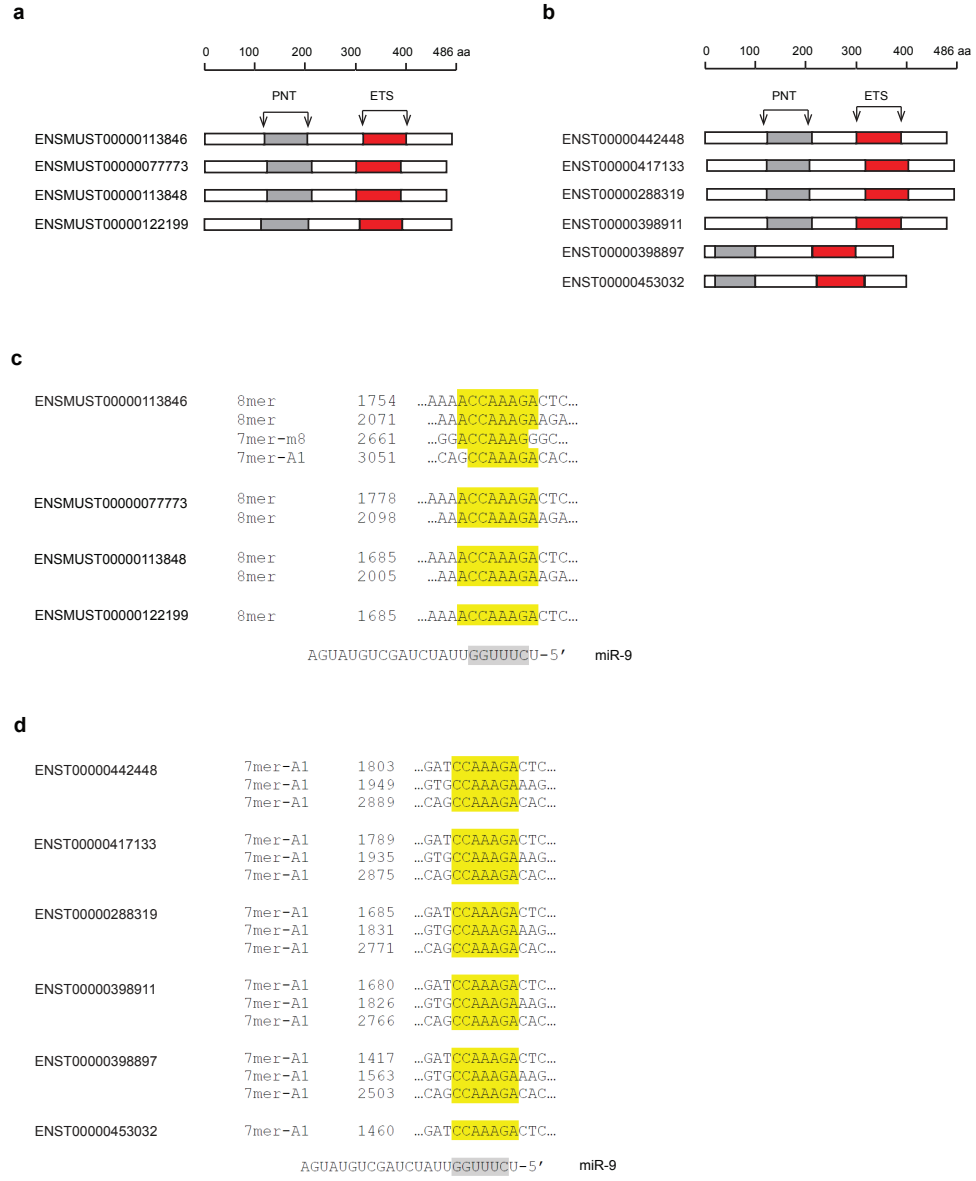

Figure S5. Known murine and human ERG isoforms. Schematic structure of known (**a**) murine and (**b**) human ERG isoforms with their important functional domains. Names are given according to transcript/protein ID in Ensembl database. PNT – pointed domain. (**cd**) MiR-9 binding sites in known (**c**) murine *Erg* and (**d**) human *ERG* transcripts (highlighted in yellow) and miR-9 seed sequence (highlighted in grey). Position of a binding site is given from the beginning of a 5′ UTR of a specific transcripts.

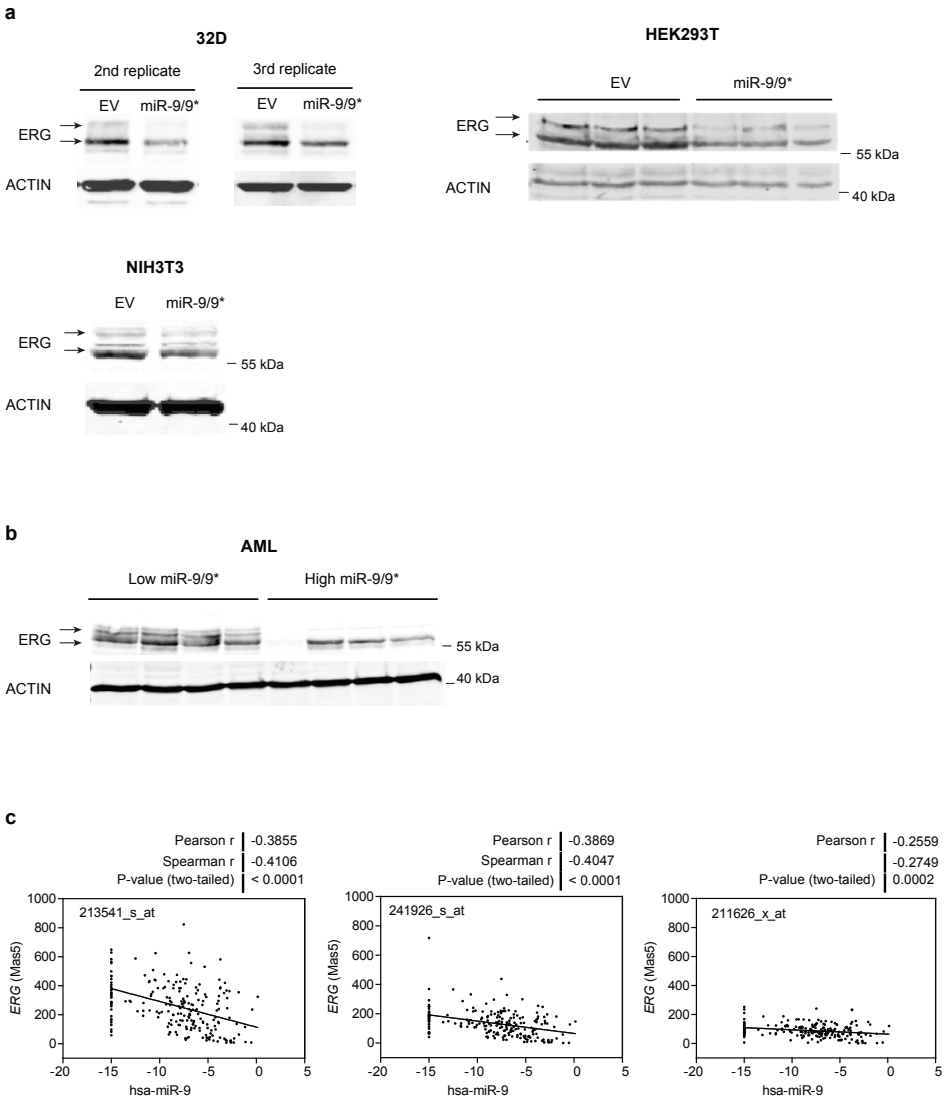

Figure S6. ERG expression upon upregulation of miR-9/9* in different cell lines, and in AML with low or high miR-9/9* levels. (**a**) Western blot analysis of different cell lines transduced with MSCV-EV (EV) or MSCV-miR-9/9* (miR-9/9*). (**b**) Western blot analysis of AML samples with low or high miR-9/9* expression level. (**c**) Correlation of miR-9 and *ERG* expression in AML. Plots show the expression of *ERG* measured with three different probe sets derived from Human Genome U133 plus 2.0 arrays.

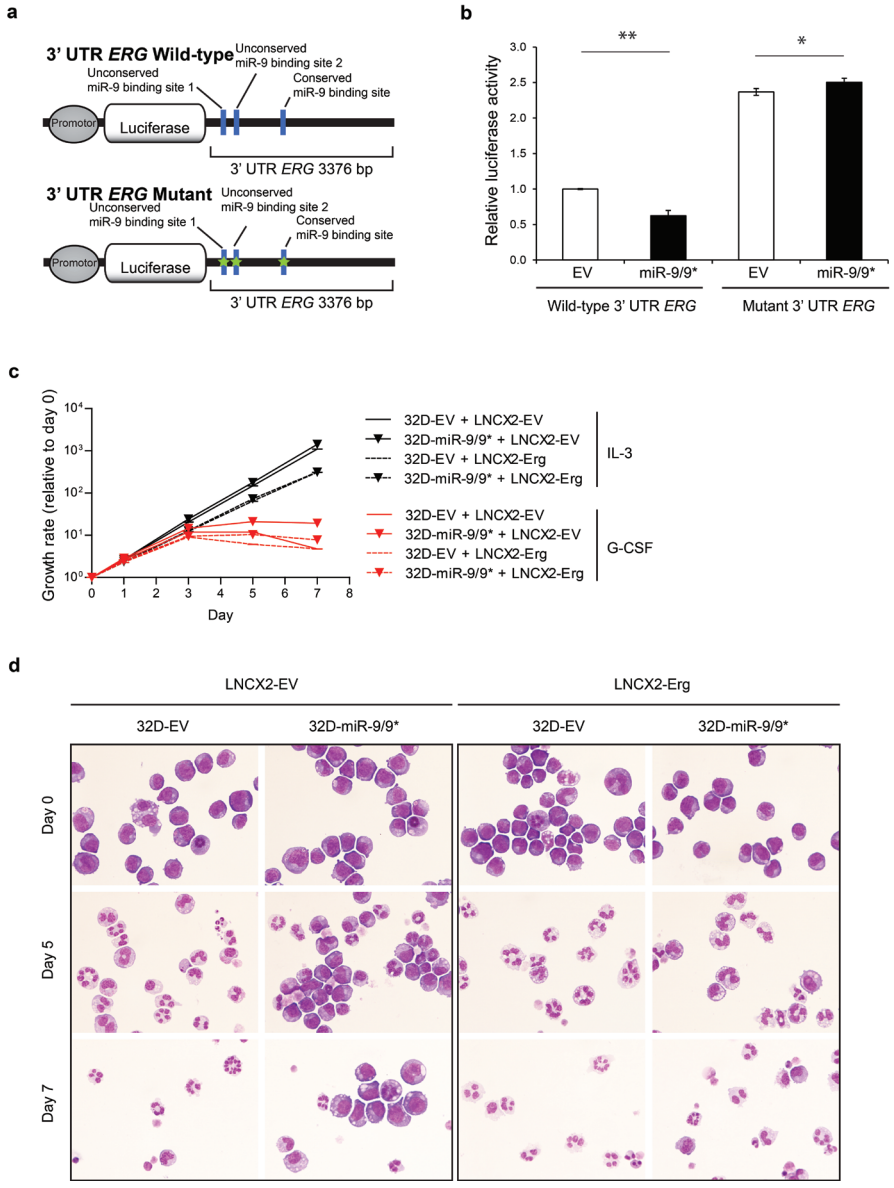

Figure S7. ERG is a direct target of miR-9 and its upregulation reverses block in neutrophil differentiation induced by miR-9/9* overexpression. (**a**) A representation of *ERG* 3' UTR luciferase reporter assay. Mutated miR-9 binding sites are marked with stars. (**b**) Relative luciferase activity in HEK293T cells that were transfected with the wild type or mutant 3' UTR of *ERG* in combination with EV or miR-9/9*. (**c**) Average growth rates of 32DEV (no symbol) and 32D-miR9/9* (triangle) cells that were transduced with LNCX2-EV (solid line) or LNCX2-Erg (dashed line); cultured upon IL-3 (black) or G-CSF (red) stimulation. Growth rates are given as number of cells at each time point divided by number of cells at day 0. (**d**) Representative micrographs of cytospins stained with May Grünwald Giemsa at different time points of GC-SF induced differentiation (original magnification ×100). (**c-d**) Data show the second of the two independent biological replicates.

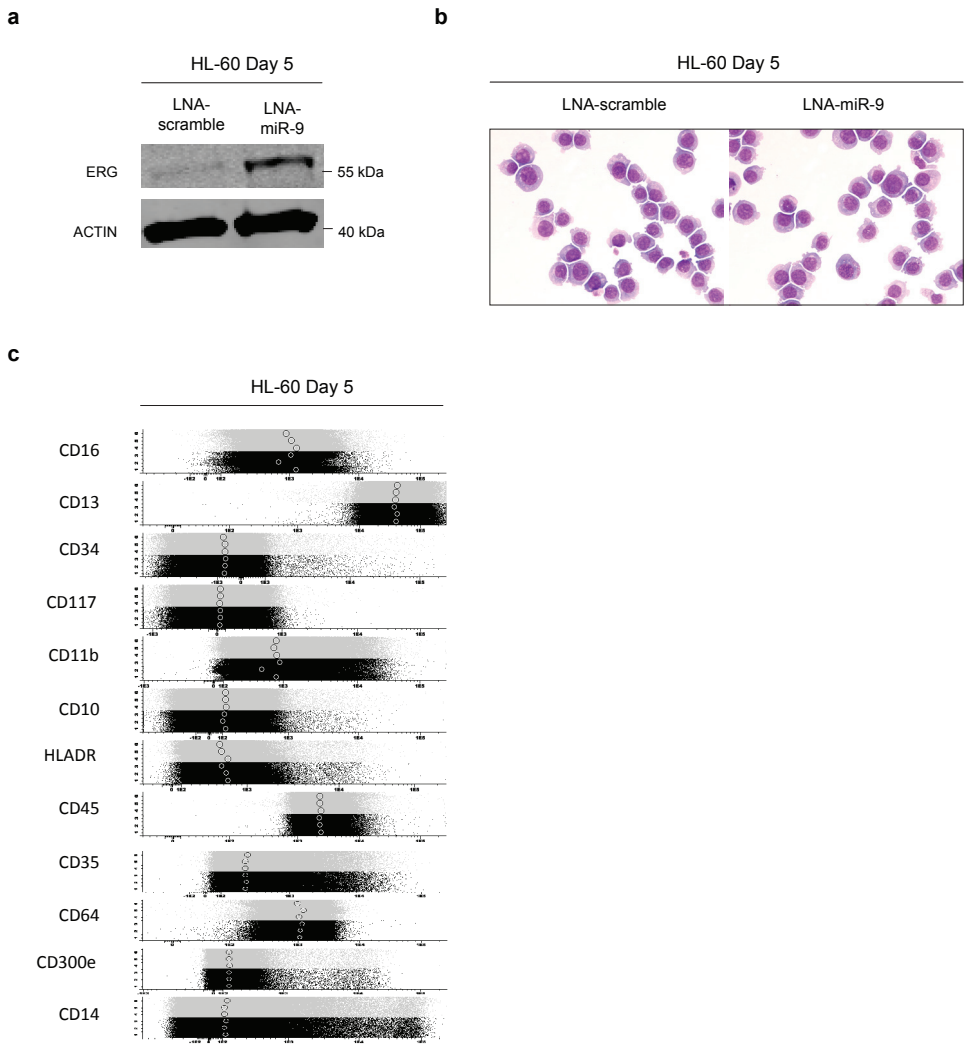

Figure S8. MiR-9 knockdown in HL-60 AML cell line model leads to upregulation of ERG expression. (**a**) Western blot analysis of HL-60 cells transfected with LNA-scramble or LNA-miR-9 at day 5. (**b**) Representative micrographs of cytospins stained with May Grünwald Giemsa at day 5 of LNA treatment (original magnification ×100). (**c**) Flow cytometric analysis of neutrophil and monocytic differentiation markers according to EuroFlow protocols at day 5 of LNA treatment. Cells treated with LNA-scramble are marked in grey and cells treated with LNA-miR-9 are marked in black. Circles represent median values of fluorescence intensity. All experiments were performed in three independent replicates.

3

EXPRESSION OF MIR-9/9* IN 32D CELLS INDUCES PROTEOME CHANGES RELATED TO CELL DIFFERENTIATION, APOPTOSIS, MIGRATION AND ADHESION

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Unpublished data

ABSTRACT

MiR-9 and miR-9* (miR-9/9*) are both aberrantly expressed in most cases of acute myeloid leukemia (AML). In a previous study, we showed that overexpression of miR-9/9* in normal myeloid progenitor cells inhibits neutrophil differentiation. Here, we investigate the proteome changes in murine myeloid 32D cells that ectopically express miR-9/9*. We found 29 proteins to be significantly down- or upregulated in a steady state, and 42 upon induction of neutrophil differentiation. The differentially expressed proteins were related to cell differentiation, apoptosis, migration and adhesion. Among the downregulated proteins, there were 5 potential miR-9/9* targets: MYO1C, ANXA2, VCL, MYH9 and ITGA6.

INTRODUCTION

MiRNAs are short non-coding RNAs (20-25 nt) that post-transcriptionally regulate gene expression.[1] They bind with their 5' regions at position 2-8 to complementary sequences within the 3' untranslated regions (3' UTRs) of target mRNAs. In this way, they act as a guide for ribonucleoprotein complexes, called miRISCs, for recognition of mRNA targets. As a result of miRISC-mRNA interaction, the mRNA undergoes decay and/or translational repression that lead to decrease in the protein level. Changes in miRNA expression may only partially influence each individual target but significantly affect all targets as a class.[2] Each miRNA can target hundreds of transcripts, therefore miRNAs are commonly parts of co-regulatory networks of direct and in direct targets that function together to influence phenotypic outcome.[3,4]

MiR-9 and miR-9* (miR-9/9*) are two miRNAs that originate from the same precursor miRNA, pre-miR-9.[5] In a previous study, we reported the aberrant expression of these miRNAs in acute myeloid leukemia (AML).[6] MiR-9/9* are not expressed in normal murine hematopoietic stem and progenitor cells and overexpression of these miRNAs in the murine myeloid 32D cell line inhibited neutrophil differentiation. Here, we investigate the effects of miR-9/9* overexpression on proteome changes in 32D cells in a steady state (IL-3) and during granulocyte colony-stimulating factor (G-CSF) induced differentiation.

MATERIALS AND METHODS

Cell culture and transduction

The mmu-miR-9-2 precursor (together with ~250 bp flanking sequence) was cloned into a pMSCV retroviral expression system containing GFP.[7] Retroviral particles were produced as previously described.[7] Cells were transduced using RetroNectin (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol and selected for GFP expression using FACSAria cell sorter (BD Biosciences, Breda, the Netherlands).

The 32D cell line was cultured as previously described in medium containing interleukin 3 (IL-3) or for 48 hours in medium containing G-CSF.[7,8]

Real-time quantitative RT-PCR

Total RNA isolation and real-time quantitative RT-PCR were performed as previously described.[7,9] Briefly, miR-9 and miR-9* expression was determined using real-time quantitative RT-PCR assays for miRNAs (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). MiR-9/9* expression was determined using sno202 as loading control. A minimal threshold for Ct values above 35 were set to a dCt value of -13. The relative quantification method $2^{-(dCt)}$ was used to calculate the relative expression.

Stable isotope labeling by amino acids in cell culture (SILAC)

32D cells that were transduced with vector containing miR-9/9* (32D-miR-9/9*) or with empty vector (32D-EV) were stably labeled with heavy amino acids [$^{13}C_6$]-L-Lysine (*Lys) and [$^{13}C_6$, $^{15}N_4$]-L-Arginine (*Arg) using SILAC™ Protein ID & Quantitation Media Kit (Thermo Fischer Scientific, Bleiswijk, the Netherlands) according to manufacturer's protocol. Full incorporation of heavy amino acids and arginine-to-proline conversion artifacts was validated after 6 doubling times using nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS). In the forward experiment, 32D-miR-9/9* were grown in RPMI medium supplemented with 10% dialyzed FBS and heavy amino acids. Simultaneously, 32D-EV were grown in medium containing regular (light) amino acids. After six doubling times, cells were harvested, mixed in a 1:1 ratio (based on cell numbers) and lysed in RIPA buffer (Sigma-Aldrich, Zwijndrecht, the Netherlands). Subsequently, SDS-PAGE and LC-MS/MS were performed as previously described.[10] In the reversed experiment, 32D-miR-9/9* were grown in medium containing light amino acids and 32D-EV in medium containing heavy amino acids. Data obtained from forward and reversed experiments were analyzed using MaxQuant and Perseus software (www.maxquant.org). A list of significantly changed proteins was generated based on the normalized protein expression ratio < 0.8 (log_2 = (0.322)) for downregulated or > 1.2 (log_2 = (0.263)) for upregulated proteins and significance B < 0.05. All experiments were performed in 2 independent biological replicates, each including 2 technical replicates (forward and reversed; total n = 4).

Ingenuity Pathway Analysis (IPA, www.ingenuity.com) was used to determine overrepresented pathways and functions that were related to differentially expressed proteins. Potential miR9/9* targets were selected using TargetScan 4.2 (www.targetscan.org) web resource.

Gene expression profiling analysis

Gene expression profiles (GEP) of 32D-miR-9/9* or 32D-EV were derived using Mouse Genome 430 2.0 array (Affymetrix, High Wycombe, UK) as published previously.[6] Data are available at www.ncbi.nlm.nih.gov/geo (GSE41942). Down- and upregulated proteins that were identified in SILAC experiments were analyzed for their mRNA expression. All experiments were performed in 3 independent replicates.

RESULTS

Proteome changes in 32D cells upon miR-9/9* overexpression

MiR-9/9* are not expressed in wild type 32D cells.[6] To investigate the effects of miR-9/9* expression on the proteome, we transduced 32D cells with a retroviral construct containing GFP and miR-9/9* or GFP only. Subsequently, cells were FACS sorted and cultured in medium containing IL-3 and light or heavy amino acids under SILAC conditions (**Figure 1a**; see Materials and Methods). Ectopic expression of miR-9/9* under light and heavy culture conditions was confirmed by real-time quantitative RT-PCR (**Figure 1b**). In all 4 independent replicates, a total of 3043 proteins were identified (**Figure 2a**). Of those, 18 were significantly downregulated and 11 were upregulated upon miR-9/9* expression (**Figure 2b**). Among these proteins, 14 were also differentially expressed on the mRNA transcript level as shown by GEP analysis (n = 3; **Figure 2c**).

To further characterize miR-9/9* expressing 32D cells, we performed Ingenuity pathway analysis. The twenty-eight differentially expressed proteins could be related to the categories of functions, such as cell death and survival (14 proteins, $P = 3.9\times10^{-4} – 6.4\times10^{-3}$), cellular movement (14 proteins, $P = 1.2\times10^{-5} – 2.1\times10^{-3}$), cell-to-cell signaling and interaction (8 proteins, $P = 4.9\times10^{-6} – 1.4\times10^{-4}$) (**Table 1**). Among differentially expressed proteins, there were 4 predicted downregulated miR-9 targets: MYO1C, ANXA2, VCL and MYH9 (**Figure 2a**, red dots). No predicted miR-9* targets were found to be downregulated.

Proteome changes in 32D cells upon miR-9/9* overexpression during induction of differentiation

32D cells are capable to differentiate from immature myeloblasts towards neutrophils within 5 days of G-CSF stimulation.[6,8] We previously showed, that miR-9/9* overexpression in 32D cells inhibits neutrophil differentiation.[6] To investigate the influence of miR-9/9* expression on the proteome during early differentiation, we transferred 32D-miR-9/9* and 32D-EV cells to media containing G-CSF and light or heavy amino acids for 48 hours and performed SILAC experiments. Overall, 2793 proteins were identified in 4 independent replicates (**Figure 3a**). In total, 24 proteins were significantly downregulated and 18 were upregulated upon miR-9/9* expression (**Figure 3b**). We found that 31 proteins were also differentially expressed on the transcript level (n = 3; **Figure 3c**).

The Ingenuity pathway analysis revealed that the differentially expressed proteins could be related to the categories of functions, such as cell death and survival (27 proteins, $P = 6.6\times10^{-9} – 4.1\times10^{-8}$), cellular movement (24 proteins, $P = 7.0\times10^{-13} – 5.3\times10^{-7}$), cellular development (20 proteins, $P = 1.5\times10^{-7}$), cell-to-cell signaling and interaction (12 proteins, $P = 7.8\times10^{-10} – 2.8\times10^{-6}$) (**Table 2**). Among downregulated proteins, we identified 3 predicted miR-9 targets: ITGA6, VCL and ANXA2 (**Figure 3a**, red dots). Similarly to the IL-3 culture condition, no predicted miR-9* targets were found.

DISCUSSION

We previously reported that miR-9/9* are aberrantly expressed in AML. Overexpression of these miRNAs in normal hematopoietic stem and progenitor cells inhibited neutrophil differentiation.[6] Since miRNAs post-transcriptionally regulate gene expression,[1,2] we investigated the effects of miR-9/9* overexpression on the proteome in murine myeloid 32D cell line.

In a stady state (IL-3 conditioned medium), ectopic expression of miR-9/9* in 32D cells significantly changed the expression of 28 proteins. Subsequent pathway analysis revealed that the proteome changes induced by overexpression of miR-9/9* could be related to increase in apoptosis (score = 1.5), decrease in migration of hematopoietic cells (score = (-1.7) – (-1.2)), and decrease in adhesion of cells (score = (-2.2)) (**Table 1**). After induction of neutrophil differentiation with G-CSF, ectopic expression of miR-9/9* significantly changed the expression of 42 proteins. The downregulated and upregulated proteins could be related to decrease in apoptosis (score = (-0.5)), migration (score = (-1.9) – (1.3)), adhesion (score = (-1.5)) and differentiation (score = (-0.5)) (**Table 2**). The larger number of influenced proteins upon G-CSF stimulation could be potentially explained by the inhibition of neutrophil differentiation in 32D-miR-9/9* as compared to 32D-EV that normally differentiate.[6] It is further supported by the pathway analysis showing that deregulated proteins relate to decrease in differentiation in miR-9/9* overexpressing cells.

In this study, we identified 5 potential miR-9/9* targets that are implicated in cellular movement and adhesion: MYO1C, ANXA2, VCL, MYH9 and ITGA6. MiR-9 was previously reported to influence migration and adhesion of cells in different human tumors. In osteosarcoma (OS), miR-9 was shown to repress genes involved in migration, differentiation and focal adhesion, including *MYO1C*, *ITGA6* and *VCL*.[4] In hepatocellular carcinoma (HCC), overexpression of miR-9 inhibited migration of carcinoma cell lines.[11] Additionally, miR-9 expression level was inversely correlated with the level of *ANXA2* gene in primary HCC tissues and *ANXA2* was shown to be a direct target of miR-9.[11]

Our results suggest that the aberrant expression of miR-9/9*, alongside blocking neutrophil differentiation, may affect apoptosis, migration and adhesion of normal myeloid cells. Further functional studies are necessary to show the potential contribution of miR-9/9*-induced proteome changes to aberrant apoptosis, migration and adhesion of normal hematopoietic stem and progenitor cells.

Acknowledgments

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Authorship

Contribution: K.N. and S.M.S. planned, carried out the experiments and analyzed the data. J.A.A.D and M.K.D. performed experiments. S.J.E., B.L. and M.J.L. designed the study and interpreted the results. K.N., S.J.E., B.L. and M.J.L. wrote or contributed to the manuscript.

Table 1. Top functions annotated to differentially expressed proteins in miR-9/9* overexpressing 32D cells upon IL-3 treatment

Category	Functions annotation	*P*	Score†	No. of proteins‡	Protein symbol§
Cell Death and Survival	cell death	3.9E-04	1.6	14	MYH9, GNG2, S100A4, ANXA2, ITGAL, SERPINB9B, POR, CSF2RB, MPO, ANXA1, PNP, ZYX, VCL, ARL11
	necrosis	5.1E-03	1.5	10	CSF2RB, MPO, ANXA1, GNG2, S100A4, PNP, ZYX, ANXA2, ITGAL, ARL11
	apoptosis	6.4E-03	1.5	10	POR, CSF2RB, MPO, ANXA1, GNG2, S100A4, PNP, ANXA2, VCL, ARL11
Cellular Movement	migration of cells	1.2E-05	0.3	13	SERPINB1, MYH9, S100A4, ANXA2, MYO1C, ITGAL, CSF2RB, IFITM3, MPO, ANXA1, ZYX, VCL, MYL12A
	cell movement of tumor cell lines	5.4E-04	2.0	7	IFITM3, MYH9, ANXA1, S100A4, ZYX, ANXA2, VCL
	chemotaxis of cells	2.5E-04	-0.4	6	SERPINB1, ANXA1, S100A4, ANXA2, BIN2, ITGAL
Tissue Morphology	quantity of cells	4.0E-03	-0.8	8	SERPINB1, CSF2RB, MYH9, MPO, ANXA1, PNP, VCL, ITGAL
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response	leukocyte migration	1.2E-04	-1.2	8	SERPINB1, CSF2RB, MYH9, MPO, ANXA1, S100A4, ANXA2, ITGAL
	cell movement of myeloid cells	2.1E-03	-1.3	5	SERPINB1, ANXA1, S100A4, ANXA2, ITGAL
	migration of phagocytes	9.0E-04	-1.7	4	MPO, ANXA1, ANXA2, ITGAL
Cell-To-Cell Signaling and Interaction	adhesion of tumor cell lines	4.9E-06	-2.2	6	MYH9, ANXA1, ZYX, ANXA2, VCL, ITGAL
	binding of cells	1.4E-04	-1.6	6	CSF2RB, MYH9, MPO, ANXA2, VCL, ITGAL
Inflammatory Response	inflammatory response	2.3E-03	-0.7	6	SERPINB1, MPO, ANXA1, S100A4, ANXA2, ITGAL

†Activation score provides an information whether experimentally observed protein expression is associated with a literature-derived direction of regulation, which can be either "activating" (score > 0) or "inhibiting" (score < 0).
‡Number of differentially expressed proteins that were annotated to a particular function.
§Predicted miR-9/9* targets that were downregulated upon miR-9/9* overexpression are marked in red.

Table 2. Top functions annotated to differentially expressed proteins in miR-9/9* overexpressing 32D cells after 48 hours of G-CSF treatment

Category	Functions annotation	*P*	Score†	No. of proteins‡	Protein symbol§
Cell Death and Survival	cell death	4.1E-08	-0.9	25	HSD17B10, FLNB, CSTA, PRDX5, IL1RL1, CTSG, S100A4, NFKB1, SUN2, ITGB3, TGM2, CASP6, HK2, S100A8, VCL, PGRMC1, LCN2, ITGA6, VIM, ANXA2, ITGAL, ITGAM, S100A9, PRTN3, NCF2
	necrosis	6.6E-09	-1.7	23	HSD17B10, CSTA, FLNB, IL1RL1, LCN2, S100A4, ITGA6, CTSG, VIM, ANXA2, NFKB1, ITGAL, SUN2, ITGB3, TGM2, CASP6, HK2, ITGAM, S100A9, PRTN3, NCF2, S100A8, PGRMC1
	apoptosis	1.2E-08	-0.5	23	HSD17B10, CSTA, FLNB, IL1RL1, PRDX5, LCN2, S100A4, ITGA6, CTSG, VIM, ANXA2, NFKB1, SUN2, ITGB3, TGM2, CASP6, HK2, ITGAM, S100A9, PRTN3, NCF2, S100A8, VCL
	cell survival	1.5E-08	1.8	17	CCR1, HSD17B10, LCN2, S100A4, VIM, NFKB1, ITGAL, PREP, TGM2, CASP6, ITGAM, HK2, S100A9, NCF2, S100A8, VCL, PGRMC1
Cellular Movement	migration of cells	9.9E-13	-1.2	24	CCR1, FLNB, LCP1, IL1RL1, LCN2, S100A4, ITGA6, CTSG, VIM, ANXA2, NCF4, NFKB1, ITGAL, SUN2, ITGB3, TGM2, ITGAM, S100A9, PRTN3, NCF2, ANXA3, TRPV2, S100A8, VCL
	chemotaxis of cells	1.0E-11	-1.9	14	CCR1, LCP1, LCN2, CTSG, ITGA6, S100A4, ANXA2, ITGAL, ITGB3, ITGAM, S100A9, PRTN3, TRPV2, S100A8
	cell movement of tumor cell lines	5.3E-07	0.2	12	TGM2, FLNB, LCP1, S100A9, S100A4, ITGA6, CTSG, VIM, S100A8, ANXA2, VCL, ITGB3
Cellular Development	differentiation of cells	1.5E-07	-0.5	20	CCR1, CSTA, FLNB, LCP1, IL1RL1, LCN2, CTSG, ITGA6, S100A4, VIM, ANXA2, NFKB1, ITGAL, ITGB3, PREP, TGM2, ITGAM, PRTN3, S100A8, ACADM
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response, Tissue Development	leukocyte migration	7.0E-13	-1.3	18	CCR1, LCP1, IL1RL1, LCN2, CTSG, ITGA6, S100A4, ANXA2, NCF4, NFKB1, ITGAL, ITGB3, TGM2, ITGAM, S100A9, PRTN3, TRPV2, S100A8
	cell movement of myeloid cells	1.3E-11	-1.2	14	CCR1, IL1RL1, LCN2, CTSG, S100A4, ANXA2, ITGAL, ITGB3, TGM2, ITGAM, S100A9, PRTN3, TRPV2, S100A8
	migration of phagocytes	1.3E-07	-1.9	8	CCR1, ITGAM, S100A9, CTSG, S100A8, ANXA2, ITGAL, ITGB3
	accumulation of neutrophils	1.0E-10	-1.6	7	ITGAM, S100A9, PRTN3, LCN2, ITGA6, CTSG, S100A8
Inflammatory Response	inflammatory response	1.2E-12	-1.6	17	CCR1, LCP1, IL1RL1, PRDX5, LCN2, CTSG, S100A4, ANXA2, NFKB1, ITGAL, ITGB3, TGM2, ITGAM, S100A9, PRTN3, TRPV2, S100A8
Tissue Morphology	quantity of cells	9.4E-05	0.6	14	CCR1, IL1RL1, LCN2, ITGA6, NFKB1, ITGAL, ITGB3, PREP, TGM2, ITGAM, S100A9, PRTN3, S100A8, VCL
Cell-To-Cell Signaling and Interaction	binding of cells	7.8E-10	-0.9	12	CCR1, TGM2, ITGAM, S100A9, PRTN3, ITGA6, CTSG, TRPV2, ANXA2, VCL, ITGAL, ITGB3
	adhesion of tumor cell lines	2.8E-06	-1.5	7	TGM2, ITGAM, ITGA6, ANXA2, VCL, ITGAL, ITGB3
Cancer, Organismal Injury and Abnormalities	metastasis	8.5E-05	1.9	9	TGM2, IL1RL1, LCN2, S100A4, ITGA6, VIM, CST7, ITGAL, ITGB3

†Activation score provides an information whether experimentally observed protein expression is associated with a literature-derived direction of regulation, which can be either "activating" (score > 0) or "inhibiting" (score < 0).
‡Number of differentially expressed proteins that were annotated to a particular function.
§Predicted miR-9/9* targets that were downregulated upon miR-9/9* overexpression are marked in red.

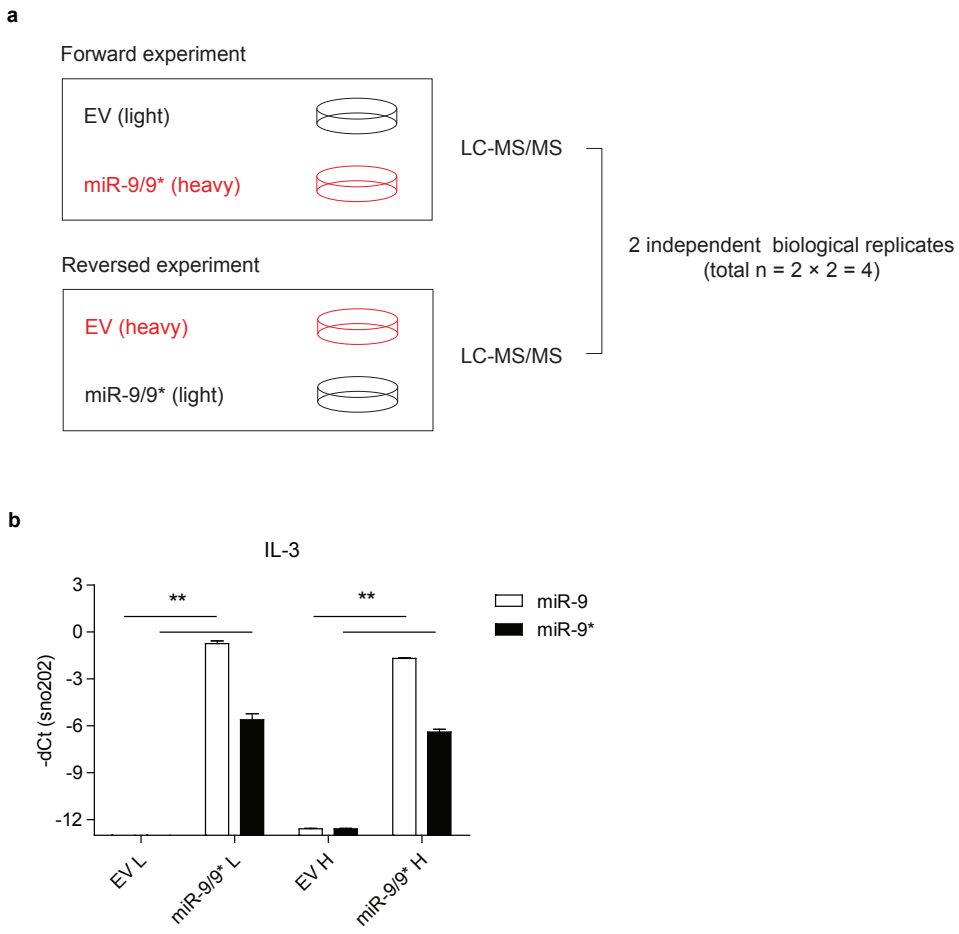

Figure 1. SILAC experimental strategy and overexpression of miR-9/9* in 32D cell line. To investigate the influence of miR-9/9* expression on the proteome changes in 32D cell line, cells were transduced with MSCVEV (EV) or MSCV-miR9/9* (miR9/9*) retroviral construct, FACS sorted, and cultured in media containing light (black) or heavy (red) amino acids. (**a**) A summary of SILAC experimental strategy. LS-MS/MS – nanoflow liquid chromatography tandem mass spectrometry. All experiments were performed in 2 independent biological replicates, each including 2 technical replicates: forward and reversed (total n = 4). (**b**) Quantitative RTPCR of miR-9 and miR-9* in cells that were transduced with empty vector (EV) or with vector containing miR-9/9* precursor (miR-9/9*) and grown in medium containing light (L) or heavy amino acids (H). Sno202 was used as endogenous control. Expression is shown as -dCt, where higher values represent higher expression. Error bars represent the standard deviation. Unpaired two-tailed t-test was used for the statistical analysis. $^{**}P < 0.001$.

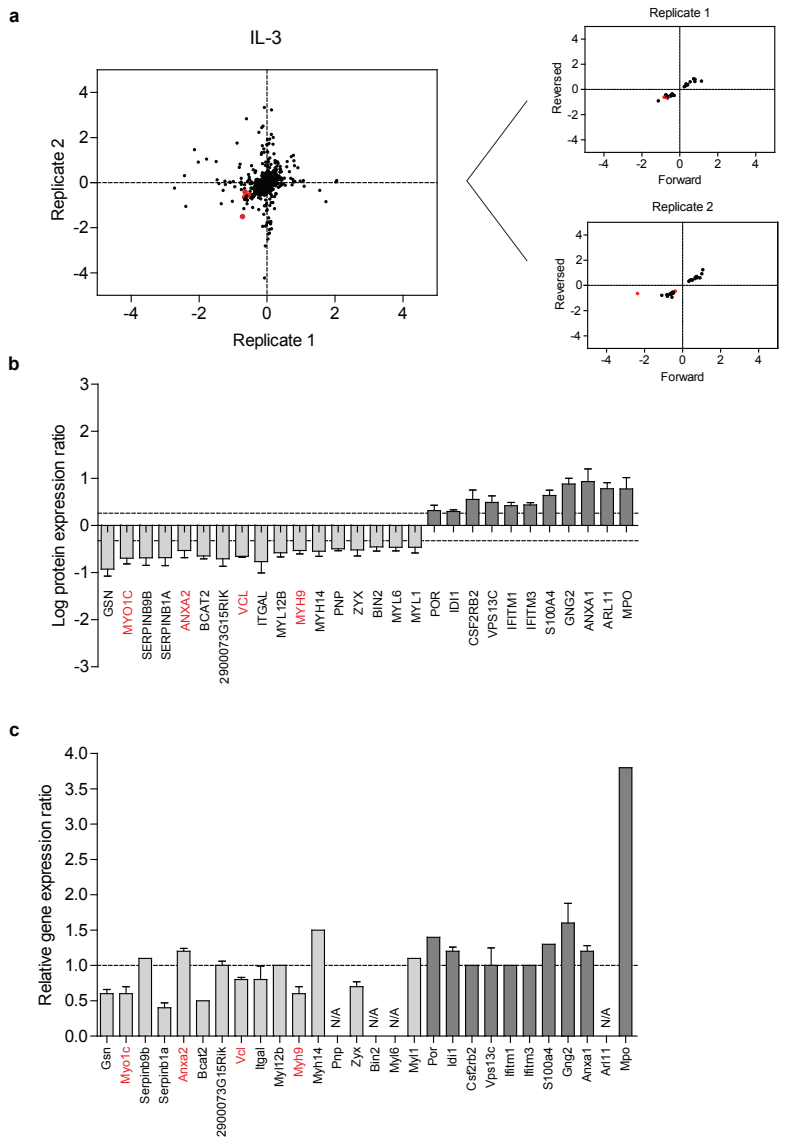

Figure 2. Proteome changes in 32D cells upon miR-9/9* overexpression. 32D cells were transduced with MSCVEV or MSCVmiR9/9* retroviral construct, FACS sorted, and cultured in media containing IL-3 upon SILAC conditions. Subsequently, a mass spectrometric analysis was performed. (**a**) A plot summarizing all identified proteins in 2 biological replicates, each including 2 technical replicates. Horizontal and vertical axes represent logarithms of normalized protein expression ratios. Proteins that were upregulated upon miR-9/9* overexpression are situated in the upper right corner and those that were downregulated in the bottom left. Significantly changed proteins upon miR-9/9* expression are shown in detail per replicate. (**b**) A plot showing the logarithms of normalized protein expression ratios of significantly upregulated or downregulated proteins upon miR-9/9* expression. (**c**) mRNA transcript levels of differentially expressed proteins. Gene expression ratios were calculated relative to empty vector in 3 independent replicates. Error bars represent the standard deviation. Potential downregulated miR-9/9* targets are marked in red.

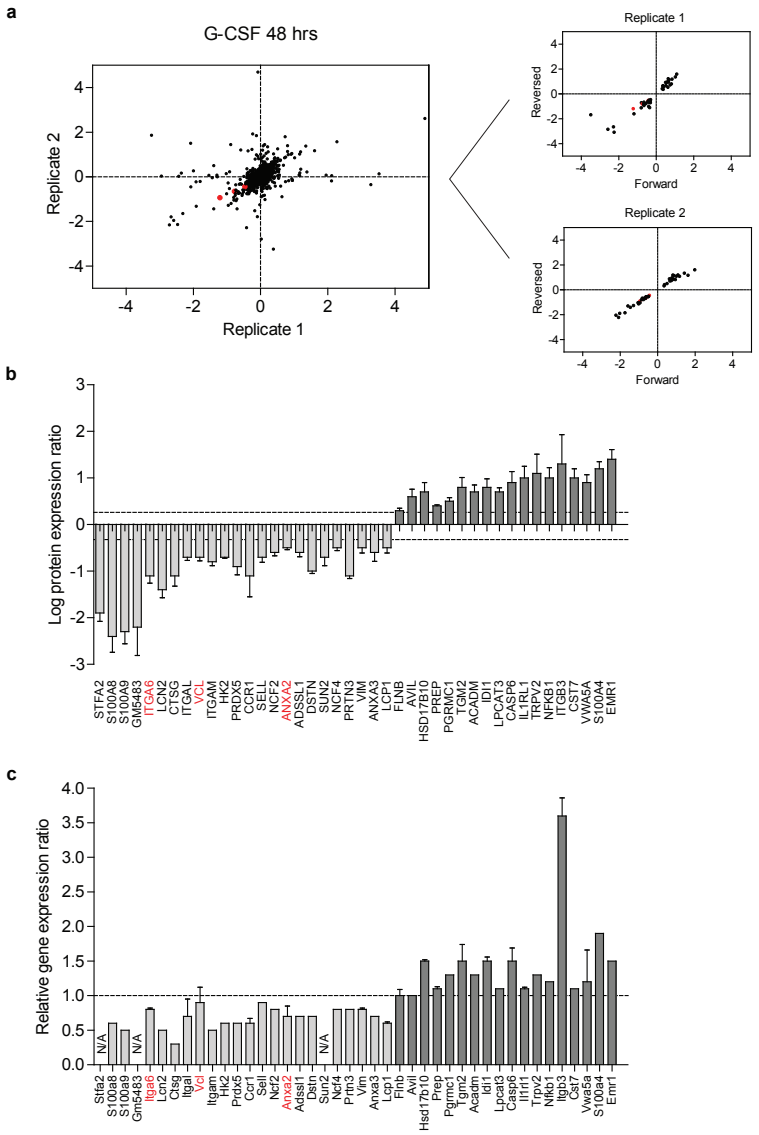

Figure 3. Proteome changes in 32D cells upon miR-9/9* overexpression during induction of differentiation. 32D cells were transduced with MSCVEV or MSCVmiR9/9* retroviral construct, FACS sorted, and cultured in media containing granulocyte colony-stimulating factor (G-CSF) for 48 hours upon SILAC conditions. Subsequently, a mass spectrometric analysis was performed. (**a**) A plot summarizing all identified proteins in 2 biological replicates, each including 2 technical replicates. Horizontal and vertical axes represent logarithms of normalized protein expression ratios. Proteins that were upregulated upon miR-9/9* overexpression are situated in the upper right corner and those that were downregulated in the bottom left. Significantly changed proteins upon miR-9/9* expression are shown in detail per replicate. (**b**) A plot showing the logarithms of normalized protein expression ratios of significantly upregulated or downregulated proteins upon miR-9/9* expression. (**c**) mRNA transcript levels of differentially expressed proteins. Gene expression ratios were calculated relative to empty vector in 3 independent replicates. Error bars represent the standard deviation. Potential downregulated miR-9/9* targets are marked in red.

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4

HEMATOPOIETIC STEM AND PROGENITOR CELLS THAT EXPRESS MIR-9/9* HAVE A DECREASED POTENTIAL TO HOME TO THE BONE MARROW

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Unpublished data

ABSTRACT

MiR-9/9* are highly conserved miRNAs that are aberrantly upregulated in most cases of acute myeloid leukemia (AML). We previously showed that aberrant expression of miR-9/9* in normal hematopoietic stem and progenitor cells (HSPCs) inhibits myeloid differentiation *in vitro* and leads to proteome changes related to differentiation, apoptosis, migration and adhesion. Here we report that in bone marrow transplantation experiments in mice, miR-9/9* overexpressing HSPCs had a reduced homing ability. We observed no differences in apoptosis and proliferative potential of these cells. Our preliminary data suggest that expression of miR-9/9* impairs different aspects of normal hematopoietic cells function.

INTRODUCTION

Hematopoiesis is a lineage-specification process that involves step-wise maturation of hematopoietic stem and progenitor cells (HSPCs). The ability of these cells to differentiate and to contribute to normal hematopoiesis depends on their clonogenic properties and the interaction with the bone marrow (BM) compartment.[1] Different hematopoietic cell types express a unique spectrum of adhesion and signaling molecules that convey their propensity to migrate and to interact with stromal cells in the hematopoietic niche.[2] Several epigenetic factors have been shown to regulate function of hematopoietic cells, among which miRNAs are recognized to play a significant role.[3,4] Altered miRNA expression has been reported to contribute to the development of acute myeloid leukemia (AML), a complex heterogeneous disease characterized by the accumulation of immature myeloid cells in the bone marrow (BM).[5]

MiRNAs are short non-coding RNAs (20-25 nt) that post-transcriptionally suppress gene expression. In this way they regulate specific molecular networks in tissue development and disease.[6] MiR-9 and miR-9* (miR-9/9*) are two highly conserved miRNAs that are produced from the miR-9 precursor RNA.[7,8] Their expression and mode of action are cell-type specific and they have been shown to be deregulated in different types of tumors.[9-11] In normal human HSPCs, miR-9 is expressed at low levels and miR-9* is not detectable.[11] However, they are both aberrantly upregulated in most cases of AML.[11,12] Our preliminary experiments in myeloid 32D cell line model indicated that miR-9/9* induce changes in expression of proteins involved in cellular movement and adhesion (unpublished data, **Chapter 3** of this thesis). Here, we investigate the effect of miR-9/9* overexpression on homing of normal HSPCs in a murine *in vivo* bone marrow transplantation model.

MATERIALS AND METHODS

Cell culture and transduction

The mmu-miR-9-2 precursor (together with ~250 bp flanking sequence) was cloned into a pMSCV retroviral expression system containing GFP.[13] Murine BM cells were harvested from femurs and tibiae of 8- to 12-week-old C57BL/6 mice, enriched for lineage negative stem and progenitor cells (HSPCs), retrovirally transduced, selected for GFP expression and cultured as previously described.[11,14]

Real-time quantitative RT-PCR

Total RNA isolation and real-time quantitative RT-PCR were performed as previously described.[11] MiR-9/9* expression in HSPCs was determined using sno202 as a loading control with a minimal threshold for Ct values above 35 set to a -dCt value of -13 when a miRNA is not expressed.

Flow cytometry

Hematopoietic progenitor population Lin$^-$c-Kit$^+$Sca-1$^-$ (LK) and hematopoietic stem/progenitor population Lin$^-$Sca-1$^+$c-Kit$^+$ (LSK) were identified by staining with Biotin Mouse Lineage Panel, streptavidin-APC-Cy7, c-Kit-APC (BD Biosciences, Breda, the Netherlands) and Sca-1-PE-Cy5 (eBioscience, Vienna, Austria). Hematopoietic stem cells (HSC) Lin$^-$Sca-1$^+$c-Kit$^+$CD150$^+$CD48$^-$ and multipotent progenitor cells (MPP) Lin$^-$Sca-1$^+$c-Kit$^+$CD150$^-$CD48$^-$ were identified by staining with CD150-PerCP/Cy5.5 and CD48-PB (Biolegend, London, United Kingdom). For apoptosis analysis, cells were stained with Annexin V:PE Apoptosis Detection Kit I (BD Biosciences). Data were collected and analyzed as previously described.[11] All experiments were performed in three independent biological replicates.

Bone marrow transplantation experiments

In homing experiments, recipient mice were lethally irradiated (8.5 Gy) and tail-vein injected with 1.5×10^5 miR-9/9* (n = 8) or EV (n = 8) transduced GFP$^+$ HSPCs. Cells overexpressing miR-9/9* and EV controls were transplanted at the same time point. BM was analyzed 20 hours after transplantation. The percent of transplanted GFP$^+$ cells that had homed to BM was calculated as previously described:[2] % homing = [(A×B×C)/D]×100%, where A equals the percent of GFP$^+$ cells and B equals the percent of living cells determined by flow cytometry, C equals the total organ cellularity, and D equals the number of cells transplanted. The absolute number of GFP$^+$ cells was calculated from the formula: no. of GFP$^+$ cells = (A×B×C)/100. The results were multiplied by 4 since cells from both femurs and tibiae are assumed to represent 25% of the entire BM.[2] All animal experiments were approved by the Animal Welfare/Ethics Committee of the Erasmus University Medical Center.

Colony-forming unit (CFU) cell assays

4×10^3 HSPCs were plated in triplicate in methocult (M3231; StemCell Technologies, Grenoble, France) supplemented with IL-3 (supernatant 1/1000), IL-6 (10 ng/mL) and SCF (10 ng/mL).[13] After 7 days 5×10^4 cells were replated under the same conditions. All experiments were performed in three independent biological replicates.

RESULTS

MiR-9/9* overexpressing HSPCs home less efficiently to the BM

To investigate the influence of miR-9/9* expression on the function of hematopoietic cells *in vivo*, we transduced normal HSPCs with retroviral construct containing GFP and miR-9/9* or GFP only. Subsequently, cells were FACS sorted and ectopic expression of these miRNAs was confirmed by real-time quantitative RT-PCR (dCt: miR-9/miR-9* = (-3.0 ± 0.5)/(-6.7 ± 0.9); see Materials and Methods). The frequencies of successfully transduced LK and LSK populations were the same for miR-9/9* (n = 3; LK: 81.4 ± 2.0%; LSK: 7.1 ± 2.6%) and EV (n = 3; LK: 82.7 ± 3.5%; LSK: 6.8 ± 2.0%; **Figure 1a-b**), and further flow cytometric analysis indicated similar distribution of HSCs and MPPs (**Figure 1a**). Cells overexpressing miR-9/9* (n = 8) and EV controls (n = 8) were transplanted into lethally irradiated C57BL/6 recipient mice and the homing of GFP$^+$ cells was assessed at 20 hours post-transplantation. We observed that miR-9/9* HSPCs were able to home to the BM (**Figure 2a-c)**. However, the percent of homing (0.9 ± 0.4%) and the absolute number of GFP$^+$ cells (1246 ± 526) in miR-9/9* transplanted animals were significantly lower than those in EV controls (% homing: 2.9 ± 0.5%; no. of GFP$^+$ cells: 4438 ± 482; **Figure 2b-c**).

MiR-9/9* overexpressing HSPCs have normal level of apoptosis and colony-forming capacity

In order to gain more insight into decreased ability of miR-9/9* overexpressing HSPCs to home to the BM, we assessed early apoptosis *in vitro*. HSPCs transduced with miR-9/9* or EV were FACS sorted and the level of apoptosis was evaluated at 0 and 24 hours after transduction. We observed no differences in frequencies of Annexin V$^+$ populations between miR-9/9* HSPCs (n = 3; 0 hours: 30.2 ± 3.5%; 24 hours: 25.0 ± 5.0%) and EV control cells (n = 3; 0 hours: 31.8 ± 5.0%; 24 hours: 26.4 ± 7.3%; **Figure 3a-b**). Next, we performed CFU assays to explore the effect of aberrant expression of miR-9/9* on colony-forming capacity of normal HSPCs. Cells overexpressing miR-9/9* and EV controls were plated in triplicate in methocult containing IL-3, IL-6 and SCF. After 7 days, there were no differences in colony numbers and size between miR-9/9* and EV transduced cells (n = 3; colony numbers per 1000 cells plated: miR-9/9* – 50.6 ± 7.8, EV – 63.9 ± 10.3; **Figure 3c-d**). Additionally, both formed no colonies after replating (**Figure 3c**). Stable overexpression of miR-9/9* in time was confirmed by real-time quantitative RT-PCR (**Figure 3e**).

DISCUSSION

In a previous study, we reported that miR-9/9* are aberrantly upregulated in human AML and that ectopic expression of miR-9/9* in normal murine HSPCs inhibits myeloid differentiation *in vitro*.[11] In addition, we observed that miR-9/9* induce changes in expression of proteins involved in cellular movement and adhesion in myeloid 32D cell line model (unpublished data, **Chapter 3** of this thesis). This provides the rationale for further investigation of the effect of miR-9/9* overexpression on the function of normal HSPCs *in vivo*.

The ability of hematopoietic cells to contribute to normal hematopoiesis relate to their potential to interact with the BM compartment. Here, we report that HSPCs that overexpressed miR-9/9* seed in the BM three times less efficiently than EV control cells. This reduction in the ability of the hematopoietic cells to home may be potentially explained by the increased apoptosis and/or decreased proliferation. However, we observed no differences in apoptosis and colony-forming potential between miR-9/9* and EV transduced cells. This indicates that the decreased homing ability may be a result of aberrant cell migration and adhesion. Overexpression of miR-9/9* in 32D cells results in proteome changes related to reduced cellular movement and adhesion (unpublished data, **Chapter 3** of this thesis). Furthermore, miR-9 has been previously reported to influence cell migration and adhesion in different types of tumors, e.g. in hepatocellular carcinoma[9] and osteosarcoma.[10] Additional experiments are needed to evaluate whether the identified proteome changes contribute to decreased migration, adhesion and *in vivo* homing of primary murine HSPCS and whether they would affect the ability of normal cells to reconstitute hematopoiesis *in vivo.*

In conclusion, these preliminary data suggest that expression of miR-9/9* impairs different aspects of normal hematopoietic cell function.

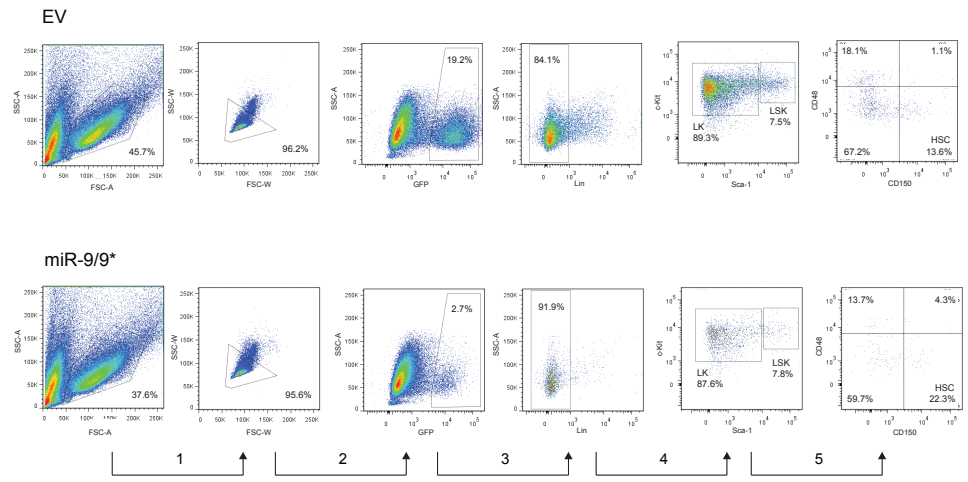
Acknowledgments

This study was supported by Erasmus MC grant and Dutch Cancer Society grant (EMCR2009-4472 to M.J.L.). We would like to thank Prof. dr. H.R. Delwel for his mentorship and contribution to interpretation of the data.

Authorship

Contribution: K.N. planned, carried out the experiments and analyzed the data. S.R.H performed experiments. E.M.J.B., S.J.E., B.L. and M.J.L. designed the study and interpreted the results. K.N., S.J.E., B.L. and M.J.L. wrote or contributed to the manuscript.

a



b

	EV		miR-9/9*	
	Mean	SD	Mean	SD
LK	82.7	3.5	81.4	2.0
LSK	6.8	2.0	7.1	2.6

Figure 1. Flow cytometric analysis of cell populations in bone marrow transplantation assay. (**a**) Representative plots of flow cytometric analysis of HSPCs that were transduced with EV or miR-9/9*. Arrows below the graphs show the direction of gating. (**b**) Summary of frequencies of LK and LSK populations in HSPCs that were transduced with EV or miR-9/9*. All experiments were performed in three independent biological replicates. Unpaired two-tailed *t*-test was used for statistical analysis.

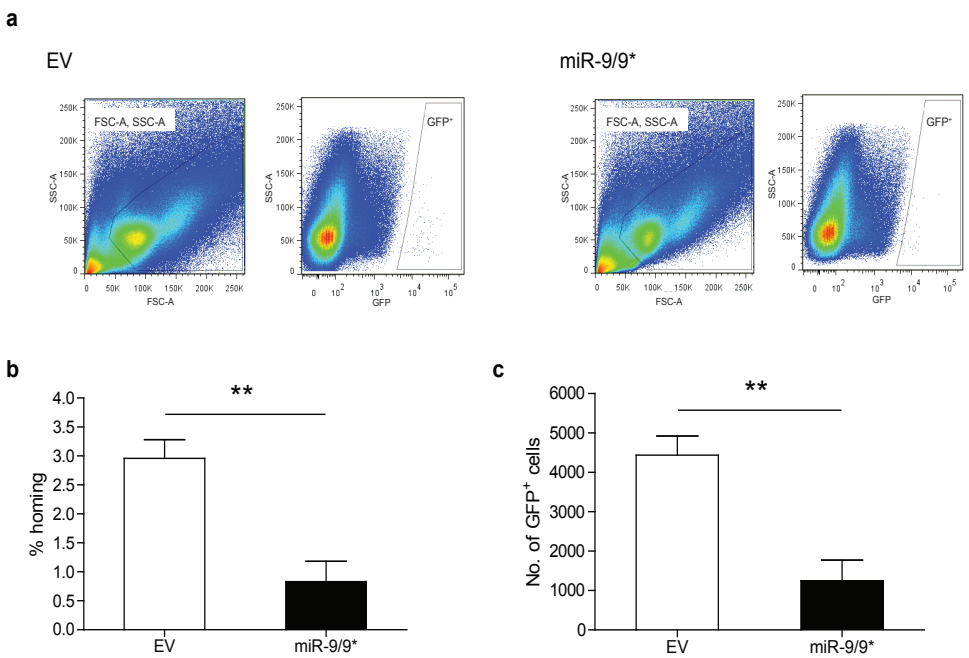


Figure 2. MiR-9/9* overexpressing HSPCs home less efficiently to the BM. (**a**) Representative plots of flow cytometric analysis of HSPCs that were transduced with EV or miR-9/9* at 20 hours post-transplantation in homing experiment. (**b-c)** A summary of (**b**) percent of homing and (**c**) a number of GFP+ cells that were found in the BM of lethally irradiated animals transplanted with EV (n = 8) or miR-9/9* (n = 8) transduced HSPCs at 20 hours post-transplanationt in homing experiment. Unpaired two-tailed *t*-test was used for statistical analysis. $^{**}P < 0.001$.

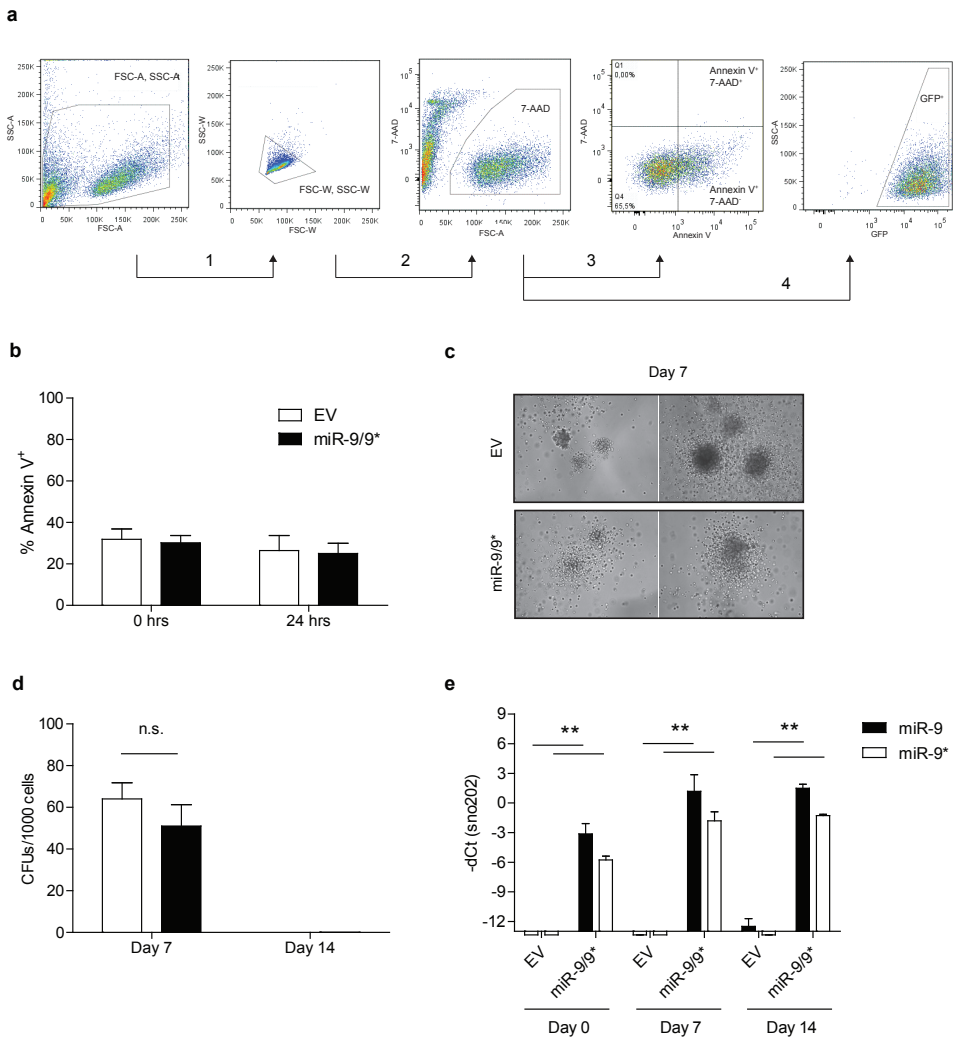


Figure 3. MiR-9/9* overexpressing HSPCs have normal level of apoptosis and colony-forming capacity. (**a**) Gating strategy of flow cytometric analysis for defining Annexin V+ population. One biological replicate is shown at 24 hours after transduction. (**b**) A summary of Annexin V+ populations in all replicates at 0 and 24 hours after transduction with EV or miR-9/9* expression construct. (**c**) Representative micrographs of colonies at day 7 of CFU assay. (**d**) A summary of colony numbers (CFUs) per 1000 cells plated at day 7 and day 14 of CFU assays. (**e**) Expression of miR-9/9* measured by real-time quantitative RT-PCR in HSPCs that were transduced EV or with vector containing miR-9/9* precursor in CFU assays. Expression level was measured post-transduction at day 0, day 7 and day 14. Sno202 was used as a loading control. Expression is shown as-dCt, where higher values represent higher expression. All experiments were performed in three independent biological replicates. Unpaired two-tailed *t*-test was used for statistical analysis. $^{**}P < 0.001$**.**

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5

EXPRESSION OF A PASSENGER MIR-9* PREDICTS FAVORABLE OUTCOME IN ADULTS WITH ACUTE MYELOID LEUKEMIA LESS THAN 60 YEARS OF AGE

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ABSTRACT

In double-stranded miRNA/miRNA* duplexes, one of the strands represents an active miRNA whereas another, known as a passenger strand (miRNA*), is typically degraded. MiR-9* is not detectable in normal myeloid cells. Here, we show that miR-9* is expressed in 59% of acute myeloid leukemia (AML) cases, and we investigate its clinical impact in 567 adults with *de novo* AML (age ≤ 60 years). AML cases with detectable miR-9* included a lower percentage of cases with favorable risk (P < 0.001) as compared to those with no detectable miR-9*. High levels of miR-9* expression independently predicted for higher CR (OR = 1.28, P = 0.013), and better EFS (HR = 0.86, P = 0.001), RFS (HR = 0.84, P = 0.008) and OS (HR = 0.86, P = 0.002). Among the subgroup of adverse risk patients, high miR-9* expressers had strikingly longer median survival than low miR-9* expressers (EFS: 16 vs. 5 months, P = 0.020; RFS: 12 vs. 4, P = 0.060; OS: 23 vs. 8, P = 0.021). Comparative transcriptome analysis suggests that miR-9* regulates genes involved in leukemogenesis, e.g., *MN1* and *MLLT3*. This is the first report showing that a miRNA* has prognostic value in AML.

INTRODUCTION

MiRNAs are short non-coding RNAs that specifically bind to their target mRNAs and decrease protein levels by destabilization of transcripts or inhibition of translation.[1] In this way they regulate crucial cellular mechanisms, such as cell differentiation and survival.[2-4] MiRNAs are expressed as primary miRNAs (pri-miRNAs) that undergo stepwise maturation.[1] Those transcripts are processed in the nucleus and in the cytoplasm, what results in the formation of double-stranded miRNA/miRNA* duplexes. At steady state, the two strands are asymmetrically expressed.[5-6] One of them, referred as miRNA, is loaded into RNA-induced silencing complex (RISC), whereas another, known as the passenger miRNA (miRNA*), is typically degraded. Nevertheless, miRNA* can be stabilized and become functional.[6] There is still relatively little known about the functions of miRNA*s in hematopoiesis but they have been proposed to have complementary roles to their related miRNAs.[6-7]

In recent years, the role of miRNAs in acute myeloid leukemia (AML) has been extensively investigated. Several miRNA-expression profiling studies have revealed that miRNA expression patterns can discriminate between different cytogenetic subtypes.[8-10] Particular miRNAs have been shown to be associated with clinical outcome. For example, high expression of miR-181a and low expression of miR-155 correlate with improved survival in cytogenetically normal AML.[11-12] Recently, we have reported about miR-212,[13] which was associated with better outcome independently of cytogenetic subtype. The prognostic relevance of miRNA*s expression in AML remains unexplored.

The three genes of *MIR9-1*, *MIR9-2* and *MIR9-3* are located at chromosomes 1, 5, and 15, respectively. They encode a highly conserved miRNA-9-5p (miR-9) and its passenger strand, miRNA-9-3p (miR-9*).[14] In normal myeloid cells, miRNA-9 is expressed at low levels, whereas miR-9* is not detectable.[15-16] Recently, we have reported that miR-9 and miR-9* are both aberrantly upregulated in AML.[16] Additionally, miR-9 has been shown to contribute to the development of *MLL*-rearranged leukemia[17] while the function of miR-9* remains unknown. Here, we report on the clinical significance of the expression of miR-9 and its passenger strand, miR-9*, in a large series of newly diagnosed well characterized patients with AML.

MATERIALS AND METHODS

Patient, treatment, and cytogenetic analysis

All 567 patients (age ≤ 60 years) included in this study had newly diagnosed AML according to the 2001 WHO classification and provided written informed consent in accordance with the Declaration of Helsinki. Patient recruitment was performed according to protocols from the Dutch-Belgian-Hematology-Oncology-Cooperative group (HOVON trials 4/4A,[18] 29,[19] 42/42A[20]; www.hovon.nl) and the German-Austrian AML Study Group (AMLSG trial HD98A).[21] All trials had been approved by the Institutional Review Board of the Erasmus University Medical Center and the University of Ulm. Sample processing and mutational analysis has been previously described.[13]

Real-time quantitative RT-PCR

Total RNA isolation and real-time quantitative RT-PCR were performed as previously described.[8,22] Briefly, miR-9 and miR-9* expression was determined in a singleplex manner using real-time quantitative RT-PCR assays for miRNAs (Applied Biosystems, Nieuwekerk a/d IJssel, the Netherlands). Data was normalized using RNU24 with a minimal threshold for Ct values above 35 set to a -dCt value of -15. MiR-9 and miR-9* expression was determined centrally at one location. The relative quantification method $2^{-(dCt)}$ was used to calculate the relative expression.[23] The expression data was log transformed to obtain symmetrical distribution.

Gene expression profiling

Gene expression profiles (GEP) of 242 AML cases were derived from Human Genome U133 plus 2.0 arrays as previously described.[24] Raw microarray data were processed using MAS5 to target intensity of 100. Intensity values lower than 30 were set to 30 and subsequently log2 transformed. GEP data are available at www.ncbi.nlm.nih.gov/geo (GSE6891). To identify differentially expressed genes, two-sided t-test analysis was performed controlling for the false discovery rate (FDR) by Benjamini-Hochberg procedure (FDR < 0.05). The analysis was done using GenePattern platform www.broadinstitute.org/cancer/software/genepattern/).[25] Ingenuity systems was used to determine overrepresented pathways and functions www.ingenuity.com/). Potential miRNA targets were selected using TargetScan 5.2 www.targetscan.org/) web resource.

Definition of clinical endpoints and statistical analysis

Complete remission (CR), event-free survival (EFS), relapse-free survival (RFS), and overall survival (OS) were defined according to the recommendations of European LeukemiaNet (ELN) guidelines.[26] Genetic risk groups were distinguished according to the ELN criteria with one minor modification concerning *CEBPA* mutations, i.e., only cases with biallelic mutations in *CEBPA* (instead of any *CEBPA* gene mutation) were considered favorable. Favorable risk group includes inv(16)(p13.1q22), t(16;16)(p13.1;q22), t(8;21)(q22;q22), cytogenetically normal karyotype (NK)-AML with mutation in *NPM1* without *FLT3*-ITD mutation, and NK-AML with biallelic mutations in *CEBPA*. Intermediate-I group contains the remainder of the NK-AML. Intermediate-II consists of t(9;11)(p22;q23) and various other cytogenetic abnormalities not classified as favorable or adverse. Inv(3)(q21q26.2) or t(3;3)(q21;q26.2), t(v;11q23), t(6;9)(p23;q34), -5 or 5q-, -7, abn(17p), and complex karyotype are designated as adverse risk. Only young adults (age ≤ 60) with primary AML and without t(15;17) were included into the analysis.

In univariable and multivariable analysis, logarithm of miRNA expression was used as a continuous linear variable. Cox proportional hazards model was used to evaluate the association of miRNA expression with EFS, RFS, and OS. The association with CR was tested using logistic regression. Known prognostic factors and non-correlated variables, that were significant in univariable analysis at P ≤ 0.20, were included into multivariable model. Age and logarithm of white blood cell count (WBC) were used as continuous linear variables, whereas ELN prognostic risk and treatment protocol as categorical variables. The proportional-hazards assumption was tested using scaled Schoenfeld residuals. Linearity of miRNA expression function and an optimal cutoff were assessed using martingale residuals.[27-28] The optimal cutoff was close to commonly used cutoff of 75% expression value, therefore patients were dichotomized at the 75th percentile of miRNA expression. Kaplan-Meier plots were used to illustrate the predictive value of miRNA, and log-rank test was used to test for differences in survival distributions.

Mann-Whitney test was used to determine the association between two continuous variables. Fisher's exact and chi-square tests were used for categorical variables. All statistical tests were two-sided. The analysis was done using Stata/Se v11.1 (College Station, TX, USA).

RESULTS

MiR-9* is differentially expressed in AML subsets

We assessed miR-9 and miR-9* expression in AML. Measurable levels of miR-9 are present in 89% of AML cases (median -dCt: -7.9, standard deviation: 3.7; within the expressers median -dCt: -7.5, standard deviation: 3.0), whereas miR-9* is measurably expressed in 59% (median -dCt: -11.5, standard deviation: 3.3; within the expressers median -dCt: -9.7, standard deviation: 2.3) (**Figure 1a; Supplementary Figure 1a-b; Supplementary Table 1**). Furthermore, miR-9* expression appears highly correlated with that of miR-9 (Pearson r = 0.779, P < 0.001; **Supplementary Figure 1c**). According their clinical and molecular characteristics, AML cases with measurable levels of miR-9* (miR9*-positive; n = 336) are different than those with no expression (miR-9*-negative; n = 231) (**Table 1**). MiR9*-positive included a smaller number of cases with favorable risk (27% vs. 44%, P < 0.001), but more intermediate-I risk cases of AML (40% vs. 21%, P < 0.001), as compared to miR-9*-negative. There were no differences in distribution of miR-9*-negative and miR-9*-positive among intermediate-II and adverse risk groups. Furthermore, miR-9*-negative AMLs included 92% (33/36) of all t(8;21) cases, and 90% (28/31) of all cases with biallelic mutations in *CEBPA*. On the other hand, miR-9*-positive subset included almost all AMLs with t(9;11) (92%, 11/12), and AMLs with mutation in the gene *NPM1* (82%, 128/156). The underlying differences in patient composition make it difficult to directly compare miR-9*-positive and miR-9*-negative cases with respect to patient outcome. MiR-9*-positive subgroup included mostly unfavorable risk AMLs. However, the comparative analysis revealed that miR-9*-positive cases had similar OS as miR-9*-negative (**Supplementary Figure 2a**). This suggested that miR-9* expression may have a positive impact on patient outcome.

MiR-9* is independently associated with better outcome in AML

To investigate the clinical impact of miR-9 and miR-9* expression in AML, we performed a univariable analysis in the patient cohorts that express those miRNAs. MiR-9 expression had no prognostic value, whereas miR-9* was significantly associated with improved EFS (hazard ratio (HR) = 0.889, 95% confidence interval (CI) = 0.815-0.969, P = 0.007), and OS (HR = 0.868, 95% CI = 0.791-0.951, P = 0.002) (**Supplementary Table 2**). Additionally, patients with AML expressing measurable miR-9* had a trend for a better CR rate (odds ratio (OR) = 1.159, 95% CI = 0.976-1.376, P = 0.092), and prolonged RFS (HR = 0.906, 95% CI = 0.806-1.018, P = 0.096). To illustrate the predictive value of miR-9*, Kaplan-Meier curves and log-rank test were used to test for differences in survival distributions. First, AML cases were split into quartiles based on miR-9* expression. Patient outcome improved with increasing levels of miR-9* (**Supplementary Figure 2b-d**). In order to define clinically relevant cutoff of miR-9* expression to characterize subgroup of patients with improved survival, we searched for the optimal cutoff using martingale residuals method. This analysis revealed that the optimal cutoff was close to the 75% expression value (data not shown). Therefore, AML patients were split into the low (n = 252) or high (n = 84) miR-9* group based on the 75th percentile (**Supplementary Table 3**). High miR-9* expressers had significantly better EFS (median survival: 18 vs. 9 months, P = 0.038; **Figure 1b**), OS (62 vs. 17 months, P = 0.020; **Supplementary Figure 3a**), and RFS (12 vs. 8 months, P = 0.043; **Supplementary Figure 4a**) than low miR-9* expressers. Detailed analysis among the distinct ELN prognostic risk groups showed that patients with high miR-9* have overall better outcomes (**Figure 2a-d; Supplementary Figure 3b-e**, and **Supplementary Figure 4b-e**). Patients in favorable risk group had improved EFS (P = 0.024; **Figure 2a**) and OS (P = 0.038; **Supplementary Figure 3b**). Patients in the intermediate-II risk category had better EFS (P = 0.050; **Figure 2c**) and RFS (P = 0.010; **Supplementary Figure 4d**). Among adverse risk patients, we observed improved outcomes for each of the survival end points; with median survival of 16 vs. 5 months for EFS (P = 0.020; **Figure 2d**), 23 vs. 8 months for OS (P = 0.021; **Supplementary Figure 3e**), and 12 vs. 4 months for RFS (P = 0.060; **Supplementary Figure 4e**). There were no notable differences in prognosis among intermediate-I risk group. Clinical and genetic characteristics of high and low miR-9* expressers were the same (**Supplementary Table 2**), with exception concerning cases with t(9;11) of which the majority (91%, 10/11, P < 0.001) belonged to the high miR-9* group.

To account for established prognostic factors and theoretical differences in treatment protocols, we performed multivariable analysis using Cox proportional hazards model for survival outcomes and logistic regression for achieving complete remission. MiR-9*, age, WBC, ELN prognostic risk, and treatment protocol were included into the model. The overall test of proportional-hazards assumption was not significant (data not shown). MiR-9* was independently predictive for better CR rate (OR = 1.284, 95% CI = 1.054-1.563, P = 0.013), as well as better EFS (HR = 0.861, 95% CI = 0.786-0.944, P = 0.001), better RFS (HR = 0.843, 95% CI = 0.744-0.956, P = 0.008) and better OS (HR = 0.855, 95% CI = 0.775-0.943, P = 0.002) (**Table 2**).

Biological insights of miR-9* expression in AML

To further characterize miR-9*-positive AML, we performed transcriptome analysis of miR-9*-positive and miR-9*-negative cases. Gene expression profiles revealed 548 probe sets, corresponding to 454 genes, to be differentially expressed (**Supplementary Table 4**). Ingenuity pathway analysis subsequently showed that these genes could be related to the categories of diseases or functions, such as cancer ($P = 9.4\times10^{-3} – 1.6\times10^{-9}$), cell death and survival ($P = 9.2\times10^{-3} – 1.9\times10^{-7}$), hematological system development and function ($P = 8.7\times10^{-3} – 2.7\times10^{-7}$) (**Table 3**). Among differentially expressed genes, there were 12 predicted downregulated miR-9* targets that included genes known to be involved in leukemogenesis, such as *MN1*[2932] and *MLLT3* (*AF9*).[17,33-34]

DISCUSSION

MiRNAs are short non-coding RNAs that are processed into double-stranded duplexes containing a guide miRNA and its passenger strand, miRNA*.[1] MiRNAs are functionally implicated into leukemogenesis,[24] and some of these have prognostic value.[11-13] MiRNA*s are typically degraded and there is not much known about their roles in hematopoiesis.[5-7] MiR-9* is not detectable in normal myeloid cells.[15-16] Here, for the first time, we report that the passenger miRNA, miR-9*, is expressed in AML (59% of cases) and has a positive impact on patient outcome.

The patient characteristics of miR-9*-positive and miR-9*-negative AML were significantly different. MiR-9*-positive included a lower percentage of favorable and a higher percentage of intermediate-I risk patients than miR-9*-negative. Percentages of intermediate-II and adverse risk groups were the same. The mechanisms of miRNA*s retained expression are largely unknown. Recently, it has been reported that various post-transcriptional mechanisms, such as RNA methylation, differential expression of RISC components, and expression of mRNA targets, may selectively prevent miRNA strand degradation.[35-37] It remains speculative whether or how various genetic abnormalities might contribute to differential miRNAs processing and lead to sustained miR-9* expression in particular subgroups of AML. Further studies of mechanisms of differential processing of miR9/miR9* duplex leading to miR-9* accumulation in AML may contribute to understanding of its preferential expression in unfavorable risk leukemias.

MiR-9 has been proposed to be part of 3-microRNA scoring system for prognostication in *de novo* AML as an independent marker of poor OS.[38] In our study, miR-9 was not significantly associated with any of the clinical response/survival endpoints. The reasons for discrepancy between our results and those previously reported are not evident but may be due to the significant differences in patient cohorts. Chuang *et al*. included in their study a substantial amount of elderly patients (41% of age > 60), as well as patients with acute promyelocytic leukemia (APL).[38] Older patients have a distinctly different prognosis and often do not receive or tolerate intensive treatment strategies. The current investigation focuses on young adults (age ≤ 60) with AML excluding APL, as patient outcome varies substantially as a function of age, and there are major differences in the treatment protocols between APL[39] and AML cases.[26] In contrast to miR-9, we found that its passenger strand, miR-9*, has a positive impact on patient outcome. High miR-9* expression was independently associated with increased CR rate and longer EFS, RFS, and OS. The increased CR rate and improved EFS may suggest that high miR-9* patients may be more sensitive to induction therapy. Indeed, there have been reports about the relationship of other miRNA*s and chemosensitivity in cancer, i.e., miR-21-3p[40] and miR-126-5p.[41]

We observed that patients with high miR-9* had overall better outcomes in a heterogeneous cohort of AML. Subsequent analysis of different ELN prognostic risk groups revealed that in favorable and intermediate-II groups, patients with high miR-9* expression

had generally better survival than low miR-9* expressers. We did not note any differences in outcome among the intermediate-I risk cases and for the time being the lack of prognostic value in the latter remains unexplained. In this study, the influence of miR-9* expression did not prevail over other potential prognostic factors. Interestingly, we observed large differences in survival outcomes between cases that expressed high and low miR-9* levels in adverse risk group of AML. Our results, for the first time, show that a single miRNA can have a clinical impact in adverse risk leukemias. In order to externally validate our findings, we have explored The Cancer Genome Atlas (TCGA) database that contains miRNA expression data of a different panel of AML patients. In 97 AML cases that matched our criteria (age ≤ 60 years, excluding APL), miR-9* was detected with the low number of 2 reads per million miRNA mapped on average. This discrepancy between our database and TCGA may largely be explained by the fact that miRNA sequencing is less sensitive in detecting low abundant transcripts than quantitative PCR if the depth of sequencing is not sufficient, as previously reported by Mestdagh *et al.*[42]

To gain more insight into the biological contribution of miR-9* expression on patient outcome and response to treatment, we compared transcriptomes of miR-9*-positive and miR-9*-negative cases. We found 12 downregulated predicted targets of miR-9* that were differentially expressed. These included genes known to be involved in leukemogenesis, such as *MN1*[2932] and *MLLT3* (*AF9*).[17,33-34] The relation of these findings with prognosis remains unsettled.

In conclusion, here we report that passenger miRNA, miR-9*, is expressed in 59% of AML cases, and has an independent positive prognostic value. Furthermore, this is the first report showing that a single miRNA has an impact on patient outcome in adverse risk group. Transcriptome analysis revealed that miR-9* may regulate genes involved in leukemogenesis. Our findings provide a rational for further studies of miRNA*s in AML.

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Authorship

Contribution: K.N. planned, carried out the experiments and analyzed the data. S.M.S. planned and carried out the experiments. L.B. and H.D. provided the German dataset and carried out experiments. M.K.D. performed experiments. S.J.E., H.D., L.B., B.L. and M.J.L. designed the study and interpreted the results. K.N., S.M.S., S.J.E., L.B., H.D., B.L. and M.J.L. wrote or contributed to the manuscript.

Table 1. Clinical and genetic characteristics of AML cohort by miR-9* expression status

	Whole cohort		miR-9*-negative		miR-9*-positive		
	No. of cases	%	No. of cases	%	No. of cases	%	P
Clinical parameters							
Age (mean)	43.9		42.9		44.6		
Range (min-max)	15.0-60.0		15.0-60.0		17.0-60.0		
WBC, x 10^9/L (mean)	48.1		45.3		50.0		
Range (min-max)	0.8-427.0		0.9-427.0		0.8-371.0		
Sex							
Total	567	*100*	231	*100*	336	*100*	
Male	292	*52*	125	54	167	*50*	
Female	275	*49*	106	46	169	*50*	
ELN prognostic risk†							<0.001
Favorable	193	*34*	101	44	92	*27*	<0.001
Intermediate-I	181	*32*	48	21	133	*40*	<0.001
Intermediate-II	99	*18*	45	20	54	*16*	
Adverse	94	17	37	16	57	*17*	
Cytogenetics							
+8	21	*4*	7	3	14	*4*	
-5 or -5q	3	*0.5*	1	0.4	2	*0.6*	
-7 or -7q	16	*3*	10	4	6	*2*	
-9q	12	*2*	8	4	4	*1*	
11q23‡	14	*3*	3	1	11	*3*	
t(9;11)	12	*2*	1	0.4	11	*3*	0.033
t(8;21)	36	*6*	33	14	3	*1*	<0.001
inv(3) or t(3;3)	4	*1*	1	0.4	3	*1*	
inv(16)	43	*8*	22	10	21	*6*	
NK	287	*51*	90	39	197	*59*	<0.001
Complex karyotype	57	*10*	25	11	32	*10*	
Other	62	*11*	30	13	32	*10*	
Molecular genetics in NK							
CEBPA double	31	*6*	28	12	3	*1*	<0.001
FLT3-ITD	110	*19*	24	10	86	*26*	<0.001
FLT3-TKD	16	*3*	1	0.4	15	*5*	0.004
NPM1	156	*28*	28	12	128	*38*	<0.001
Treatment protocol							<0.001
HOVON04	9	*2*	2	1	7	*2*	
HOVON04A	14	*3*	8	4	6	*2*	
HOVON29	61	11	25	11	36	*11*	
HOVON42	83	*15*	19	8	64	*19*	<0.001
HOVON42A	180	*32*	98	42	82	*24*	<0.001
HD98A	220	*39*	79	34	141	*42*	

Mann-Whitney test was used for continuous variables. Fisher's exact and chi-square tests were used for categorical variables.

†Only patients with biallelic mutations in *CEBPA* were included in favorable risk group (instead of any *CEBPA* gene mutation).

‡11q23 category contains *MLL*-related leukemias harboring 11q23 abnormalities other than t(9;11).

NOTE. Percentages may add up >100% because of rounding.

Abbreviations: AML, acute myeloid leukemia; WBC, white blood cell count; ELN, European LeukemiaNet; NK, normal karyotype; CEBPA, CCAAT/enhancer-binding protein α; FLT3-ITD, internal tandem duplications in the FMS-like tyrosine kinase 3; FLT3-TKD, FMS-like tyrosine kinase 3 with mutations in tyrosine kinase domain; NPM1, nucleophosmin.

Table 2. Multivariable analysis evaluating the clinical impact of miR-9* expression in AML

Variable	CR			EFS			RFS			OS		
	OR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
miR-9*	1.28	1.05-1.56	0.013	0.86	0.79-0.94	0.001	0.84	0.74-0.96	0.008	0.86	0.78-0.94	0.002
Age	1.00	0.98-1.03	0.914	1.00	0.99-1.02	0.664	1.01	0.99-1.04	0.185	1.02	1.00-1.03	0.033
WBC	0.76	0.59-0.96	0.022	1.21	1.09-1.35	0.001	1.23	1.06-1.43	0.007	1.29	1.14-1.44	<0.001
ELN risk†												
Favorable	5.48	2.23-13.48	<0.001	0.24	0.16-0.35	<0.001	0.20	0.12-0.35	<0.001	0.26	0.17-0.39	<0.001
Intermediate-II	0.86	0.38-1.99	0.734	0.90	0.62-1.30	0.565	0.84	0.50-1.43	0.524	0.69	0.46-1.04	0.079
Adverse	0.52	0.24-1.13	0.100	1.28	0.90-1.84	0.173	1.56	0.92-2.66	0.098	1.69	1.16-2.45	0.006
Treatment‡												
HOVON-4	0.28	0.04-1.94	0.198	1.55	0.54-4.38	0.414	1.28	0.16-9.91	0.813	1.89	0.70-5.14	0.211
HOVON-4A	0.81	0.13-5.19	0.824	1.51	0.64-3.57	0.347	3.99	1.27-12.5	0.018	1.00	0.39-2.57	0.999
HOVON-29	3.73	0.99-14.1	0.052	0.70	0.43-1.14	0.156	0.85	0.42-1.72	0.657	0.90	0.54-1.49	0.680
HOVON-42	3.99	1.37-11.7	0.011	0.56	0.37-0.85	0.008	0.65	0.35-1.21	0.179	0.57	0.35-0.88	0.012
HD98A	0.51	0.25-1.03	0.062	1.54	1.10-2.17	0.013	2.14	1.29-3.57	0.003	1.16	0.81-1.67	0.416

Multivariable logistic regression was used for CR, and Cox proportional hazards model was used for EFS, RFS, and OS. HR < 1 or HR > 1 indicate a decreased or increased risk. OR < 1 or OR > 1 indicate a decreased or increased odds for reaching CR.
†Only patients with biallelic mutations in *CEBPA* were included in favorable risk group. Intermediate-I group was used as a reference.
‡P-values determined by likelihood-ratio test of a model with and without treatment protocol were < 0.01. HOVON-42A treatment protocol was used as a reference.
Abbreviations: AML, acute myeloid leukemia; WBC, white blood cell count; ELN, European LeukemiaNet; CR, complete remission; EFS, event-free survival; RFS, relapse-free survival; OS, overall survival; OR, odds ratio; HR, hazard ratio; CI, confidence interval.

Table 3. Top diseases and functions annotated to differentially expressed genes in miR-9*-positive vs. miR-9*-negative

Category	Diseases or Functions annotation	P	Score†	No. of genes‡	↓ miR9* targets§
Cancer	cancer	6.5E-03	-1.3	318	*AKAP2, B4GALT6, GLIS3, GNAI1, IKZF2, LRP6, MAN1A1, MLLT3, MN1, RHOBTB3, SOX1*
Cell Death and Survival	cell death	2.7E-05	0.8	84	GLIS3, LRP6, MLLT3
	necrosis	4.6E-04	1.5	67	
	cell death of tumor cell lines	7.8E-03	1.7	51	
	cell death of leukemia cell lines	1.8E-06	1.3	22	
	apoptosis of colon cancer cell lines	5.8E-03	2.3	12	
	killing of cells	4.5E-03	0.9	7	
Cellular Movement	cell movement	1.6E-06	0.9	57	*GNAI1*
	migration of cells	4.5E-07	1.0	54	
	migration of carcinoma cell lines	1.5E-03	0.7	8	
	migration of hepatoma cell lines	7.2E-03	-1.4	5	
Cell Signaling, Molecular Transport, Vitamin and Mineral Metabolism	mobilization of Ca^{2+}	2.1E-05	1.8	16	*GNAI1*
Cellular Development, Hematological System Development and Function, Hematopoiesis	differentiation of leukocytes	4.6E-05	0.8	15	MLLT3
	differentiation of myeloid cells	5.8E-04	1.2	10	
Cell Cycle	cell cycle progression of tumor cell lines	3.2E-03	-1.4	12	*MLLT3*
Carbohydrate Metabolism	glycolysis	4.0E-03	-1.2	5	LRP6

†Activation score provides an information whether experimentally observed gene expression is associated with a literature-derived direction of regulation, which can be either "activating" (score > 0) or "inhibiting" (score < 0).
‡Number of differentially expressed genes that were annotated to a particular function. See also Table S3.
§Predicted miR-9* targets that were downregulated in miR-9*-positive vs. miR-9*-negative.

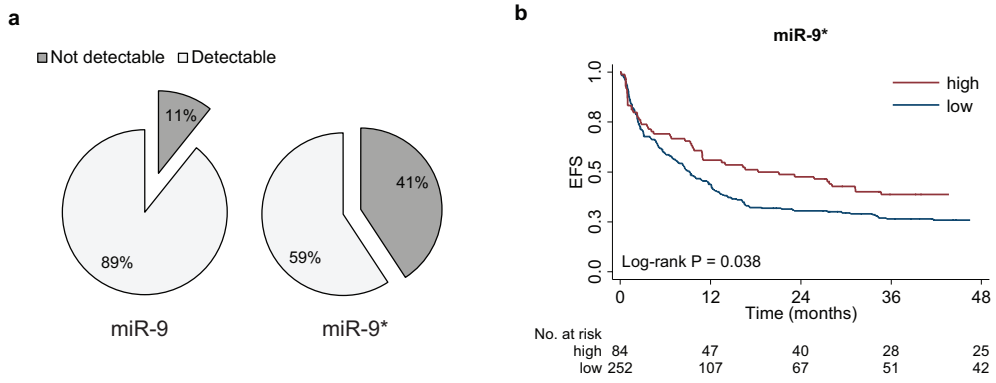

Figure 1. MiR-9* is expressed in 59% of AML cases, and it is associated with better outcome. (**a**) Percentage of AML cases that express miR-9, and miR-9*. (**b**) Event-free survival (EFS) of AML patients according to miR-9* expression. Patients were dichotomized into high and low expression groups based on the 75th percentile of miR-9* expression value (see Materials and Methods).

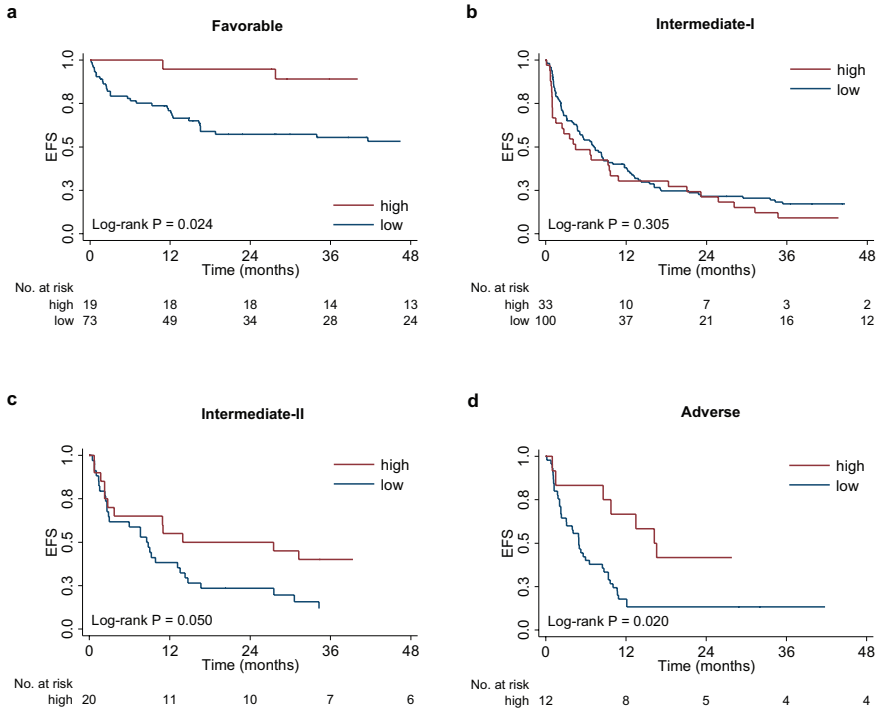

Figure 2. Patients with high miR-9* have overall better outcomes in different ELN prognostic risk groups. Event-free survival (EFS) of AML patients belonging to (**a**) favorable, (**b**) intermediate-I, (**c**) intermediate-II, and (**d**) adverse risk group according to miR-9* expression. Patients were dichotomized into high and low expression groups based on the 75th percentile of miR-9* expression value (see Materials and Methods).

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SUPPLEMENTARY DATA

Table S1. MiR-9 and miR-9* expression in patients with AML. Raw Ct data and normalized expression values (see Materials and Methods) from real-time quantitative RT-PCR.

Assay name	hsa-miR-9		hsa-miR-9*	
miRBase ID	hsa-miR-9-5p		hsa-miR-9-3p	
Target Sequence	UCUUUGGUUAUCUAGCUGUAUGA		AUAAAGCUAGAUAACCGAAAGU	
Sample	Raw Ct	Log(2^(-dCt))	Raw Ct	Log(2^(-dCt))
AML 1	27.06	-2.51	30.40	-4.82
AML 2	35.00	-11.44	35.00	-11.44
AML 3	28.90	-3.63	32.05	-5.82
AML 4	27.78	-2.84	30.71	-4.87
AML 5	34.06	-7.72	35.00	-11.44
AML 6	31.91	-6.17	34.46	-7.93
AML 7	30.29	-5.48	33.25	-7.53
AML 8	31.09	-6.03	34.45	-8.36
AML 9	30.62	-1.94	32.80	-3.45
AML 10	33.57	-7.51	35.00	-11.44
AML 11	32.78	-7.15	35.00	-11.44
AML 12	35.00	-11.44	34.71	-6.64
AML 13	35.00	-11.44	35.00	-11.44
AML 14	33.64	-5.38	34.32	-5.84
AML 15	34.50	-4.41	34.29	-4.26
AML 16	30.92	-5.01	33.20	-6.58
AML 17	35.00	-11.44	35.00	-11.44
AML 18	29.13	-3.47	31.42	-5.06
AML 19	27.35	-2.75	31.16	-5.39
AML 20	29.76	-4.04	32.52	-5.96
AML 21	26.80	-1.66	28.45	-2.80
AML 24	34.00	-4.88	35.00	-11.44
AML 25	33.91	-7.22	34.35	-7.53
AML 26	35.00	-11.44	35.00	-11.44
AML 27	29.62	-5.05	32.80	-7.25
AML 28	34.50	-7.92	35.00	-11.44
AML 29	29.62	-4.51	33.05	-6.89
AML 30	35.00	-11.44	35.00	-11.44
AML 32	28.52	-3.59	31.75	-5.83
AML 33	31.57	-4.80	34.90	-7.11
AML 34	28.93	-4.51	31.67	-6.41
AML 35	30.34	-5.48	33.61	-7.75
AML 36	30.52	-2.38	33.31	-4.32
AML 37	28.72	-3.68	31.64	-5.70
AML 38	23.24	-0.13	26.82	-2.61
AML 40	32.71	-7.17	34.23	-8.23
AML 42	27.59	-3.42	30.75	-5.61
AML 43	31.59	-6.11	34.38	-8.04
AML 44	28.80	-3.76	31.28	-5.48
AML 45	30.34	-4.92	33.58	-7.17
AML 46	33.15	-6.86	35.00	-11.44
AML 47	29.69	-4.21	33.14	-6.60
AML 48	32.23	-6.32	34.88	-8.16
AML 49	35.00	-11.44	35.00	-11.44
AML 50	34.63	-5.96	35.00	-11.44
AML 51	30.87	-5.61	33.42	-7.37
AML 52	30.67	-4.40	34.38	-6.97
AML 53	33.38	-7.26	35.00	-11.44
AML 54	35.00	-11.44	35.00	-11.44
AML 55	23.64	-0.22	27.07	-2.60
AML 56	30.45	-4.57	32.88	-6.26
AML 57	29.62	-4.51	33.05	-6.89
AML 58	33.51	-7.17	34.79	-8.06
AML 59	29.18	-4.02	32.43	-6.28
AML 60	26.46	-1.16	29.65	-3.38
AML 61	29.72	0.08	32.73	-2.00
AML 62	35.00	-11.44	35.00	-11.44
AML 63	33.69	-6.81	35.00	-11.44
AML 64	32.28	-4.48	35.00	-11.44
AML 65	31.67	-5.53	33.47	-6.78
AML 66	35.00	-11.44	35.00	-11.44
AML 67	30.26	-4.45	32.99	-6.34
AML 68	25.99	-1.66	29.00	-3.75
AML 69	26.55	-2.40	30.43	-5.09
AML 70	27.43	-3.61	30.37	-5.65
AML 72	25.09	-1.43	28.64	-3.89
AML 73	25.32	-1.55	28.58	-3.81
AML 74	29.98	-4.61	32.54	-6.38
AML 75	31.11	-5.42	34.68	-7.89
AML 76	23.47	-0.34	27.16	-2.90
AML 77	29.33	-4.40	31.92	-6.20
AML 78	26.82	-2.85	29.90	-4.99
AML 79	32.69	-6.58	35.00	-11.44
AML 80	27.64	-3.33	31.20	-5.80
AML 81	29.39	-4.07	32.72	-6.37
AML 82	26.91	-3.15	30.25	-5.46

Assay name	hsa-miR-9		hsa-miR-9*	
miRBase ID	hsa-miR-9-5p		hsa-miR-9-3p	
Target Sequence	UCUUUGGUUAUCUAGCUGUAUGA		AUAAAGCUAGAUAACCGAAAGU	
Sample	Raw Ct	Log(2^(-dCt))	Raw Ct	Log(2^(-dCt))
AML 83	30.86	-5.74	33.22	-7.38
AML 84	25.37	-1.68	28.81	-4.06
AML 85	32.40	-7.06	34.42	-8.46
AML 86	29.15	-4.09	31.74	-5.89
AML 87	30.51	-5.14	33.49	-7.20
AML 88	30.27	-5.37	32.91	-7.20
AML 89	26.24	-1.80	29.47	-4.04
AML 90	30.56	-5.11	33.29	-7.01
AML 91	31.07	-6.18	32.63	-7.26
AML 92	27.17	-2.51	29.85	-4.37
AML 93	34.20	-8.62	35.00	-11.44
AML 94	27.30	-3.41	31.67	-6.43
AML 95	28.07	-3.49	31.71	-6.01
AML 96	28.83	-3.61	32.52	-6.17
AML 97	33.23	-6.69	35.00	-11.44
AML 98	35.00	-11.44	35.00	-11.44
AML 99	22.64	0.06	26.09	-2.34
AML 100	33.62	-6.98	35.00	-11.44
AML 101	33.37	-7.41	35.00	-11.44
AML 102	23.29	2.57	26.80	0.14
AML 103	31.26	-5.72	33.42	-7.22
AML 104	33.45	-6.95	34.57	-7.73
AML 105	35.00	-11.44	35.00	-11.44
AML 106	28.91	-4.19	32.40	-6.61
AML 107	34.03	-7.30	35.00	-11.44
AML 108	30.90	-5.01	33.52	-6.83
AML 109	31.62	-6.16	35.00	-11.44
AML 110	28.65	-3.75	31.56	-5.77
AML 111	26.31	-2.30	29.86	-4.75
AML 112	26.88	-2.85	30.52	-5.37
AML 113	29.77	-4.36	32.62	-6.34
AML 114	26.98	-2.40	30.41	-4.78
AML 115	33.91	-7.59	35.00	-11.44
AML 116	32.33	-6.12	34.20	-7.42
AML 117	26.21	-1.77	29.51	-4.06
AML 118	30.14	-4.14	35.00	-11.44
AML 119	34.22	-7.14	35.00	-11.44
AML 120	35.00	-11.44	35.00	-11.44
AML 121	32.17	-5.61	35.00	-11.44
AML 122	35.00	-11.44	35.00	-11.44
AML 123	32.39	-6.56	35.00	-11.44
AML 124	34.59	-7.66	35.00	-11.44
AML 125	30.95	-5.91	34.35	-8.28

Assay name	hsa-miR-9		hsa-miR-9*	
miRBase ID	hsa-miR-9-5p		hsa-miR-9-3p	
Target Sequence	UCUUUGGUUAUCUAGCUGUAUGA		AUAAAGCUAGAUAACCGAAAGU	
Sample	Raw Ct	Log(2^(-dCt))	Raw Ct	Log(2^(-dCt))
AML 126	34.43	-7.95	35.00	-11.44
AML 128	34.39	-7.87	35.00	-11.44
AML 129	34.86	-7.54	35.00	-11.44
AML 130	35.00	-11.44	35.00	-11.44
AML 131	30.49	-5.43	33.23	-7.33
AML 132	32.67	-6.45	34.92	-8.01
AML 133	33.51	-6.90	35.00	-11.44
AML 135	33.61	-6.84	35.00	-11.44
AML 136	34.64	-7.95	35.00	-11.44
AML 137	26.28	-1.80	30.07	-4.42
AML 138	28.98	-3.94	33.04	-6.76
AML 139	33.84	-6.81	35.00	-11.44
AML 142	35.00	-11.44	35.00	-11.44
AML 144	35.00	-11.44	35.00	-11.44
AML 149	31.35	-5.63	35.00	-11.44
AML 150	31.62	-5.34	35.00	-11.44
AML 151	33.00	-6.51	35.00	-11.44
AML 152	35.00	-11.44	35.00	-11.44
AML 153	32.25	-6.65	34.86	-8.46
AML 154	35.00	-11.44	35.00	-11.44
AML 155	33.66	-6.04	33.87	-6.19
AML 156	26.04	0.00	27.63	-1.10
AML 157	24.83	-1.45	27.30	-3.17
AML 158	30.82	-3.89	33.09	-5.46
AML 159	29.38	-4.01	32.38	-6.09
AML 160	34.29	-5.95	33.68	-5.53
AML 161	28.71	-3.33	32.47	-5.94
AML 162	33.87	-7.93	35.00	-11.44
AML 163	32.11	-6.05	35.00	-11.44
AML 164	35.00	-11.44	35.00	-11.44
AML 165	26.07	-2.31	28.99	-4.33
AML 166	29.21	-4.57	32.20	-6.64
AML 167	35.00	-11.44	35.00	-11.44
AML 168	26.37	-1.21	29.76	-3.56
AML 170	32.14	-7.39	35.00	-11.44
AML 171	25.67	-2.30	28.44	-4.22
AML 172	30.88	-4.77	34.30	-7.14
AML 174	34.84	-7.36	35.00	-11.44
AML 175	34.96	-6.23	35.00	-11.44
AML 176	32.31	-5.73	34.06	-6.94
AML 179	28.67	-4.07	31.37	-5.95
AML 180	31.07	-4.90	33.31	-6.45
AML 181	35.00	-11.44	35.00	-11.44

Assay name	hsa-miR-9		hsa-miR-9*	
miRBase ID	hsa-miR-9-5p		hsa-miR-9-3p	
Target Sequence	UCUUUGGUUAUCUAGCUGUAUGA		AUAAAGCUAGAUAACCGAAAGU	
Sample	Raw Ct	Log(2^(-dCt))	Raw Ct	Log(2^(-dCt))
AML 182	33.96	-6.78	35.00	-11.44
AML 183	29.35	-5.54	30.91	-6.62
AML 184	28.93	-4.15	30.30	-5.10
AML 185	29.43	-4.70	30.98	-5.78
AML 186	31.52	-6.50	33.49	-7.87
AML 187	32.51	-7.04	33.85	-7.97
AML 188	29.23	-3.54	30.19	-4.21
AML 189	31.50	-5.97	32.59	-6.73
AML 190	31.95	-6.36	33.33	-7.32
AML 192	26.48	-2.73	28.80	-4.34
AML 193	32.15	-7.23	34.51	-8.86
AML 195	25.82	-2.89	27.80	-4.27
AML 196	29.31	-4.15	31.50	-5.68
AML 198	35.00	-11.44	35.00	-11.44
AML 199	31.06	-5.64	33.42	-7.28
AML 200	32.53	-6.56	33.99	-7.58
AML 201	35.00	-11.44	35.00	-11.44
AML 202	32.17	-6.41	33.52	-7.35
AML 203	25.89	-1.83	29.44	-4.29
AML 204	30.69	-5.23	32.37	-6.40
AML 205	35.00	-11.44	35.00	-11.44
AML 206	30.35	-4.97	31.90	-6.05
AML 207	30.53	-5.49	33.93	-7.84
AML 208	34.28	-7.68	35.00	-11.44
AML 209	34.72	-8.13	35.00	-11.44
AML 210	32.77	-6.66	34.85	-8.11
AML 211	32.15	-6.36	33.55	-7.33
AML 212	32.67	-6.73	34.34	-7.89
AML 213	30.30	-5.25	32.94	-7.08
AML 215	33.33	-7.15	35.00	-11.44
AML 216	33.70	-0.97	35.00	-11.44
AML 217	29.17	-4.70	32.20	-6.80
AML 218	35.00	-11.44	35.00	-11.44
AML 220	24.99	-1.34	27.89	-3.35
AML 221	33.82	-7.56	34.36	-7.93
AML 222	33.61	-7.12	34.81	-7.95
AML 223	31.22	-5.62	33.17	-6.97
AML 225	30.49	-5.42	32.81	-7.02
AML 226	25.41	-1.58	28.53	-3.75
AML 227	34.10	-7.31	35.00	-11.44
AML 229	30.52	-5.75	32.39	-7.05
AML 230	34.93	-8.78	35.00	-11.44
AML 231	23.97	-0.80	26.91	-2.84

Assay name	hsa-miR-9		hsa-miR-9*	
miRBase ID	hsa-miR-9-5p		hsa-miR-9-3p	
Target Sequence	UCUUUGGUUAUCUAGCUGUAUGA		AUAAAGCUAGAUAACCGAAAGU	
Sample	Raw Ct	Log(2^(-dCt))	Raw Ct	Log(2^(-dCt))
AML 232	32.91	-7.14	35.00	-11.44
AML 233	34.69	-8.31	35.00	-11.44
AML 235	26.93	-2.61	31.81	-5.99
AML 236	35.00	-11.44	35.00	-11.44
AML 237	34.93	-7.78	35.00	-11.44
AML 239	29.76	-4.95	31.50	-6.15
AML 240	34.03	-7.57	35.00	-11.44
AML 241	29.18	-5.24	31.54	-6.87
AML 242	34.18	-7.97	35.00	-11.44
AML 243	32.02	-6.78	33.88	-8.06
AML 246	34.49	-7.64	34.49	-7.65
AML 247	35.00	-11.44	35.00	-11.44
AML 249	34.68	-6.98	35.00	-11.44
AML 250	31.54	-5.69	34.11	-7.47
AML 252	35.00	-11.44	35.00	-11.44
AML 322	33.00	-7.66	35.00	-11.44
AML 1174	29.89	-5.44	33.41	-8.12
AML 1188	31.62	-6.55	34.54	-8.44
AML 1299	30.04	-6.25	33.44	-7.84
AML 1316	35.00	-11.44	35.00	-11.44
AML 1551	28.58	-5.52	35.00	-11.44
AML 1595	29.23	-5.55	33.52	-7.66
AML 1747	26.63	-4.31	35.00	-11.44
AML 1766	35.00	-11.44	35.00	-11.44
AML 2169	26.07	-2.08	30.18	-5.12
AML 2171	32.95	-7.13	32.59	-8.26
AML 2172	22.20	0.10	34.56	-9.25
AML 2175	28.17	-3.06	31.56	-6.68
AML 2176	29.64	-4.27	33.02	-7.60
AML 2184	25.58	-2.77	27.41	-5.78
AML 2186	35.00	-11.44	35.00	-11.44
AML 2188	27.21	-4.14	30.22	-6.20
AML 2189	31.28	-6.18	35.00	-11.44
AML 2190	33.21	-8.09	35.00	-11.44
AML 2192	35.00	-11.44	35.00	-11.44
AML 2194	32.50	-7.56	35.00	-11.44
AML 2195	32.39	-5.20	33.56	-7.87
AML 2197	35.00	-11.44	34.44	-9.74
AML 2199	26.43	-4.18	30.05	-5.94
AML 2200	33.09	-8.11	35.00	-11.44
AML 2201	29.34	-4.85	30.74	-7.08
AML 2203	26.91	-4.07	35.00	-11.44
AML 2205	29.87	-5.01	32.75	-7.81

Assay name	hsa-miR-9		hsa-miR-9*	
miRBase ID	hsa-miR-9-5p		hsa-miR-9-3p	
Target Sequence	UCUUUGGUUAUCUAGCUGUAUGA		AUAAAGCUAGAUAACCGAAAGU	
Sample	Raw Ct	Log(2^(-dCt))	Raw Ct	Log(2^(-dCt))
AML 2207	25.95	-3.59	29.08	-6.09
AML 2208	35.00	-11.44	35.00	-11.44
AML 2210	35.00	-11.44	35.00	-11.44
AML 2212	28.87	-5.16	32.73	-7.23
AML 2215	30.03	-5.57	35.00	-11.44
AML 2216	35.00	-11.44	35.00	-11.44
AML 2217	24.35	-1.62	30.56	-5.11
AML 2218	35.00	-11.44	35.00	-11.44
AML 2220	26.15	-2.60	35.00	-11.44
AML 2222	32.54	-7.42	33.56	-8.12
AML 2223	32.70	-7.69	33.79	-8.36
AML 2224	32.23	-6.08	33.89	-7.88
AML 2226	25.99	-2.84	31.60	-6.21
AML 2227	30.16	-3.83	35.00	-11.44
AML 2228	32.31	-4.68	35.00	-11.44
AML 2229	30.59	-4.54	29.85	-5.50
AML 2230	35.00	-11.44	35.00	-11.44
AML 2231	28.94	-2.69	31.02	-5.76
AML 2234	34.22	-9.38	34.14	-9.06
AML 2235	33.50	-7.53	32.53	-8.47
AML 2236	30.77	-5.33	32.29	-7.51
AML 2238	35.00	-11.44	35.00	-11.44
AML 2239	29.90	-3.81	33.93	-8.31
AML 2240	35.00	-11.44	35.00	-11.44
AML 2241	31.86	-7.23	35.00	-11.44
AML 2242	34.85	-9.01	35.00	-11.44
AML 2243	31.88	-6.21	35.00	-11.44
AML 2244	24.72	-1.82	27.95	-4.21
AML 2245	32.36	-7.28	31.97	-7.03
AML 2246	28.03	-4.55	29.68	-6.75
AML 2247	25.18	-2.57	32.65	-7.96
AML 2248	26.28	-3.83	29.78	-5.43
AML 2250	35.00	-11.44	35.00	-11.44
AML 2253	35.00	-11.44	35.00	-11.44
AML 2254	35.00	-11.44	33.85	-8.31
AML 2256	31.33	-5.45	31.70	-7.50
AML 2257	29.98	-5.44	30.94	-7.13
AML 2260	27.26	-3.57	28.05	-4.94
AML 2261	30.85	-6.30	32.79	-6.87
AML 2268	31.73	-7.65	33.40	-8.43
AML 2271	26.03	-2.71	28.97	-4.91
AML 2273	35.00	-11.44	35.00	-11.44
AML 2275	29.87	-5.02	35.00	-11.44

Assay name	hsa-miR-9		hsa-miR-9*	
miRBase ID	hsa-miR-9-5p		hsa-miR-9-3p	
Target Sequence	UCUUUGGUUAUCUAGCUGUAUGA		AUAAAGCUAGAUAACCGAAAGU	
Sample	Raw Ct	Log(2^(-dCt))	Raw Ct	Log(2^(-dCt))
AML 2276	23.55	-1.27	27.50	-4.01
AML 2278	30.89	-5.09	33.73	-7.26
AML 2283	31.22	-4.94	35.00	-11.44
AML 2285	27.95	-3.60	31.03	-6.46
AML 2286	30.84	-5.36	35.00	-11.44
AML 2288	32.08	-6.60	30.48	-5.05
AML 2289	28.77	-4.36	35.00	-11.44
AML 2291	26.33	-3.37	29.40	-4.75
AML 2292	27.48	-2.65	30.91	-5.93
AML 2296	28.76	-4.83	31.44	-6.90
AML 2326	29.13	-5.51	35.00	-11.44
AML 2327	35.00	-11.44	35.00	-11.44
AML 2747	31.89	-6.41	33.70	-8.63
AML 3308	25.25	-2.13	28.97	-4.75
AML 3309	29.26	-4.95	31.75	-6.91
AML 3310	27.18	-3.51	31.41	-5.84
AML 3311	29.34	-4.75	34.67	-9.12
AML 3312	24.61	-2.25	32.07	-5.87
AML 3313	35.00	-11.44	35.00	-11.44
AML 3314	30.43	-5.96	31.82	-6.94
AML 3316	25.39	-2.15	29.47	-5.12
AML 3319	29.16	-4.47	33.06	-7.44
AML 3321	26.61	-3.27	29.19	-5.80
AML 3322	21.75	-0.36	24.32	-2.72
AML 3323	33.76	-8.11	35.00	-11.44
AML 3324	32.46	-6.45	35.00	-11.44
AML 3326	25.08	-2.32	35.00	-11.44
AML 3327	35.00	-11.44	35.00	-11.44
AML 3482	30.69	-6.19	33.20	-7.49
AML 3483	28.38	-4.07	31.64	-6.92
AML 3484	25.02	-2.19	28.87	-5.00
AML 4333	35.00	-11.44	35.00	-11.44
AML 4334	31.08	-5.75	34.62	-8.41
AML 4335	30.74	-5.94	34.55	-7.65
AML 4336	35.00	-11.44	35.00	-11.44
AML 4337	29.99	-5.24	33.42	-7.62
AML 4338	29.86	-5.21	35.00	-11.44
AML 4339	25.86	-1.86	29.54	-4.05
AML 4340	26.55	-2.79	30.22	-4.87
AML 4341	30.54	-5.49	34.53	-7.70
AML 5282	28.49	-5.17	33.42	-7.89
AML 5283	35.00	-11.44	35.00	-11.44
AML 5284	31.97	-6.64	34.63	-9.06

Assay name	hsa-miR-9		hsa-miR-9*	
miRBase ID	hsa-miR-9-5p		hsa-miR-9-3p	
Target Sequence	UCUUUGGUUAUCUAGCUGUAUGA		AUAAAGCUAGAUAACCGAAAGU	
Sample	Raw Ct	Log(2^(-dCt))	Raw Ct	Log(2^(-dCt))
AML 5285	27.99	-4.32	31.45	-6.34
AML 5286	28.55	-5.09	33.48	-8.40
AML 5357	34.03	-9.07	35.00	-11.44
AML 5358	22.69	-0.80	27.14	-3.40
AML 5359	29.87	-5.14	33.32	-7.15
AML 5360	25.69	-2.57	29.96	-4.87
AML 5361	26.89	-3.99	31.78	-6.32
AML 5362	35.00	-11.44	35.00	-11.44
AML 5363	28.43	-3.46	31.60	-5.81
AML 5364	32.39	-7.40	35.00	-11.44
AML 5365	29.27	-4.49	33.06	-7.48
AML 6237	26.15	-3.06	30.89	-5.68
AML 6238	29.13	-4.20	32.95	-7.35
AML 6239	30.53	-5.39	33.98	-8.13
AML 6240	30.89	-7.06	34.39	-8.67
AML 6241	26.08	-2.97	31.13	-5.93
AML 6243	33.76	-8.51	35.00	-11.44
AML 6245	23.74	-1.41	28.58	-4.17
AML 6246	22.68	-0.29	26.74	-3.13
AML 6247	32.99	-6.78	34.92	-8.15
AML 6359	33.72	-6.95	34.24	-7.89
AML 6363	30.00	-4.82	33.36	-7.37
AML 6364	23.71	-0.77	27.91	-2.90
AML 6365	35.00	-11.44	35.00	-11.44
AML 6368	29.33	-4.65	32.47	-6.83
AML 6370	27.08	-3.87	32.22	-6.86
AML 6371	23.73	-1.92	27.43	-4.08
AML 6372	35.00	-11.44	35.00	-11.44
AML 6373	30.81	-5.46	33.61	-7.39
AML 6374	28.71	-4.69	33.60	-7.79
AML 6376	35.00	-11.44	35.00	-11.44
AML 6379	29.14	-5.24	33.71	-7.97
AML 6448	32.15	-5.74	35.00	-11.44
AML 7301	27.11	-3.82	31.31	-5.93
AML 7302	25.33	-2.73	30.97	-5.43
AML 7303	35.00	-11.44	35.00	-11.44
AML 7304	29.06	-4.79	32.73	-7.53
AML 7305	27.90	-3.49	31.39	-6.23
AML 7306	25.49	-2.13	30.15	-4.73
AML 7307	24.74	-2.69	29.06	-4.59
AML 7308	30.93	-6.29	33.78	-8.57
AML 7309	28.03	-4.06	31.73	-6.24
AML 7310	28.64	-4.95	33.94	-8.08

Assay name	hsa-miR-9		hsa-miR-9*	
miRBase ID	hsa-miR-9-5p		hsa-miR-9-3p	
Target Sequence	UCUUUGGUUAUCUAGCUGUAUGA		AUAAAGCUAGAUAACCGAAAGU	
Sample	Raw Ct	Log(2^(-dCt))	Raw Ct	Log(2^(-dCt))
AML 7311	29.31	-4.54	32.41	-5.77
AML 7312	29.73	-4.64	32.88	-6.43
AML 7313	33.33	-7.98	35.00	-11.44
AML 7314	29.52	-4.86	34.15	-7.64
AML 7315	30.35	-5.77	34.72	-7.56
AML 7316	29.88	-4.82	34.04	-7.75
AML 7317	29.44	-4.54	33.68	-6.51
AML 7318	31.79	-5.95	33.34	-7.43
AML 7319	26.54	-3.14	31.06	-5.72
AML 7320	26.06	-2.92	30.24	-5.02
AML 12500	30.13	-5.58	34.33	-8.50
AML 12501	33.77	-8.16	35.00	-11.44
AML 12502	33.24	-7.34	35.00	-11.44
AML 12503	32.61	-7.84	35.00	-11.44
AML 12504	25.45	-2.55	29.81	-5.57
AML 12505	26.66	-3.00	30.98	-5.99
AML 12506	27.39	-3.28	30.72	-5.59
AML 12507	34.76	-8.43	35.00	-11.44
AML 12509	34.79	-7.87	35.00	-11.44
AML 12510	32.22	-6.56	35.00	-11.44
AML 12511	26.28	-2.54	31.04	-5.84
AML 12512	31.23	-5.54	35.00	-11.44
AML 12513	29.26	-4.86	33.32	-7.66
AML 12514	28.82	-4.80	33.11	-7.76
AML 12515	28.20	-4.04	31.88	-6.60
AML 12516	33.48	-7.44	35.00	-11.44
AML 12517	31.75	-6.07	35.00	-11.44
AML 12518	33.40	-7.64	35.00	-11.44
AML 12519	29.44	-4.54	34.35	-7.94
AML 12520	29.51	-4.40	34.23	-7.67
AML 12521	28.25	-3.34	32.88	-6.55
AML 12522	32.24	-7.17	35.00	-11.44
AML 12523	28.55	-4.14	32.34	-6.77
AML 12524	31.75	-6.66	35.00	-11.44
AML 12525	29.09	-5.03	32.23	-7.21
AML 12526	25.85	-2.68	30.30	-5.76
AML 12527	34.18	-8.54	35.00	-11.44
AML 12528	27.38	-3.01	30.73	-5.34
AML 12529	26.82	-2.75	30.34	-5.19
AML 12530	29.30	-2.96	33.26	-5.70
AML 12531	34.49	-7.84	35.00	-11.44
AML 12532	32.77	-5.96	35.00	-11.44
AML 12534	32.27	-7.48	35.00	-9.37

Assay name	hsa-miR-9		hsa-miR-9*	
miRBase ID	hsa-miR-9-5p		hsa-miR-9-3p	
Target Sequence	UCUUUGGUUAUCUAGCUGUAUGA		AUAAAGCUAGAUAACCGAAAGU	
Sample	Raw Ct	Log(2^(-dCt))	Raw Ct	Log(2^(-dCt))
AML 12535	23.27	-0.95	27.12	-3.62
AML 12536	30.22	-4.60	33.39	-6.80
AML 12554	23.46	-1.21	28.04	-4.39
AML 12555	29.89	-5.36	32.35	-7.06
AML 12556	33.18	-7.13	35.00	-11.44
AML 12557	30.13	-5.34	34.59	-8.43
AML 12558	28.23	-4.45	33.76	-8.29
AML 12560	34.72	-8.88	35.00	-11.44
AML 12561	23.90	-1.03	28.28	-4.06
AML 12562	29.20	-5.28	34.98	-9.28
AML 12563	28.52	-3.56	33.60	-7.08
AML 12564	30.70	-5.39	33.81	-7.54
AML 12565	26.90	-3.06	30.65	-5.66
AML 12566	29.18	-4.85	32.69	-7.29
AML 12567	32.45	-6.55	35.00	-11.44
AML 13960	33.98	-8.53	35.00	-11.44
AML 13960	33.98	-8.53	35.00	-11.44
AML 13961	30.73	-6.23	34.85	-9.08
AML 13962	34.59	-8.58	35.00	-11.44
AML 13963	33.29	-7.73	35.00	-11.44
AML 13963	33.29	-7.73	35.00	-11.44
AML 13964	31.38	-6.39	35.00	-11.44
AML 13968	23.61	-1.59	27.84	-4.52
AML 13970	31.72	-7.14	35.00	-11.44
AML 13972	31.59	-6.74	33.97	-8.39
AML 13973	32.69	-6.64	35.00	-11.44
AML 13975	33.61	-8.13	35.00	-11.44
AML 13976	29.60	-4.84	34.78	-8.43
AML 13977	29.61	-4.76	33.38	-7.38
AML 13978	35.00	-11.44	35.00	-11.44
AML 13981	33.57	-8.17	35.00	-11.44
AML 13982	27.08	-2.80	32.23	-6.37
AML 13983	23.26	-1.36	27.57	-4.35
AML 13984	31.54	-7.27	35.00	-11.44
AML 13986	30.23	-5.45	33.88	-7.98
AML 13986	30.23	-5.45	33.88	-7.98
AML 13988	33.69	-8.48	35.00	-11.44
AML 13989	23.47	-1.13	28.15	-4.37
AML 13993	28.67	-4.34	32.84	-7.23
AML 13995	32.76	-7.32	35.00	-11.44
AML 13996	31.78	-6.05	35.00	-11.44
AML 13997	29.57	-4.65	33.97	-7.70
AML 13999	30.58	-5.71	35.00	-11.44

Assay name	hsa-miR-9		hsa-miR-9*	
miRBase ID	hsa-miR-9-5p		hsa-miR-9-3p	
Target Sequence	UCUUUGGUUAUCUAGCUGUAUGA		AUAAAGCUAGAUAACCGAAAGU	
Sample	Raw Ct	Log(2^(-dCt))	Raw Ct	Log(2^(-dCt))
AML 14001	29.44	-5.36	34.19	-8.65
AML 14003	27.40	-3.59	32.76	-7.31
AML 14004	29.71	-4.78	33.82	-7.63
AML 14005	31.71	-6.51	35.00	-11.44
AML 14006	32.19	-7.27	35.00	-11.44
AML 14007	32.65	-7.19	35.00	-11.44
AML 14008	25.94	-2.60	30.98	-6.10
AML 14009	30.81	-5.96	35.00	-11.44
AML 14010	26.40	-2.04	31.93	-5.88
AML 14011	28.57	-4.02	33.82	-7.66
AML 14012	28.18	-4.29	34.00	-8.32
AML 14013	34.68	-8.17	35.00	-11.44
AML 14014	27.90	-3.37	32.99	-6.89
AML 14016	34.43	-8.45	35.00	-11.44
AML 14017	25.71	-2.40	31.39	-6.33
AML 14018	28.58	-4.03	34.22	-7.94
AML 14019	31.41	-6.66	35.00	-11.44
AML 14020	29.83	-5.66	35.00	-11.44
AML 14021	34.67	-8.26	35.00	-11.44
AML 14022	32.54	-6.42	35.00	-11.44
AML 14023	31.30	-5.94	35.00	-11.44
AML 14024	32.13	-6.80	35.00	-11.44
AML 14025	32.10	-6.67	35.00	-11.44
AML 14026	30.23	-4.75	35.00	-11.44
AML 14027	33.20	-7.45	35.00	-11.44
AML 14028	35.00	-11.44	35.00	-11.44
AML 14030	27.41	-3.85	33.57	-8.12
AML 14031	29.02	-4.45	35.00	-11.44
AML 14032	32.20	-6.35	35.00	-11.44
AML 14033	26.94	-2.95	31.00	-5.77
AML 14034	27.63	-3.38	33.36	-7.36
AML 14035	33.39	-7.77	35.00	-11.44
AML 14036	32.41	-6.10	35.00	-11.44
AML 14037	31.83	-6.06	35.00	-11.44
AML 14039	30.79	-5.64	35.00	-11.44
AML 14296	33.83	-7.55	35.00	-11.44
AML 14298	31.08	-5.55	35.00	-11.44
AML 14300	29.11	-3.92	34.72	-7.81
AML 14302	30.64	-5.34	34.81	-8.23
AML 14304	31.41	-6.72	35.00	-11.44
AML 14306	29.94	-4.92	34.77	-8.27
AML 14307	31.19	-6.20	35.00	-11.44
AML 14308	32.73	-6.99	35.00	-11.44

Assay name	hsa-miR-9		hsa-miR-9*	
miRBase ID	hsa-miR-9-5p		hsa-miR-9-3p	
Target Sequence	UCUUUGGUUAUCUAGCUGUAUGA		AUAAAGCUAGAUAACCGAAAGU	
Sample	Raw Ct	Log(2^(-dCt))	Raw Ct	Log(2^(-dCt))
AML 14309	32.95	-7.00	35.00	-11.44
AML 14310	27.57	-2.56	32.79	-6.17
AML 14311	32.10	-6.70	35.00	-11.44
AML 14312	32.28	-7.06	35.00	-11.44
AML 14313	33.60	-7.16	35.00	-11.44
AML 14314	31.52	-5.90	35.00	-11.44
AML 14315	28.46	-4.01	34.10	-7.92
AML 14316	33.64	-7.38	35.00	-11.44
AML 14319	29.35	-4.80	33.78	-7.87
AML 14321	32.51	-6.60	35.00	-11.44
AML 14323	32.65	-4.60	35.00	-11.44
AML 14324	25.40	-1.62	30.14	-4.90
AML 14326	35.00	-11.44	35.00	-11.44
AML 14329	35.00	-11.44	35.00	-11.44
AML 14331	31.39	-5.86	35.00	-11.44
AML 14334	29.11	-4.44	35.00	-11.44
AML 14335	32.79	-6.93	35.00	-11.44
AML 14338	29.85	-5.07	34.49	-8.29
AML 14340	27.48	-2.56	33.25	-6.56
AML 14341	33.10	-7.36	35.00	-11.44
AML 14342	27.41	-2.93	32.94	-6.77
AML 14343	27.93	-3.74	33.43	-7.55
AML 14344	34.26	-7.93	35.00	-11.44
AML 14345	27.04	-2.90	31.80	-6.20
AML 14348	31.57	-6.16	35.00	-11.44
AML 14349	30.67	-6.46	35.00	-11.44
AML 14351	34.58	-7.39	35.00	-11.44
AML 14354	27.70	-3.54	32.74	-7.04
AML 14355	28.60	-3.24	33.34	-6.53
AML 14356	31.15	-5.71	35.00	-11.44
AML 14359	30.67	-6.00	35.00	-11.44
AML 14361	30.54	-5.69	35.00	-11.44
AML 14362	23.39	-0.70	29.11	-4.67
AML 14363	35.00	-11.44	35.00	-11.44
AML 14364	27.52	-3.46	32.80	-7.12
AML 14365	29.62	-5.01	35.00	-11.44
AML 14366	34.24	-8.02	35.00	-11.44
AML 14368	25.17	-1.96	30.48	-5.64
AML 14369	31.81	-6.29	35.00	-11.44

Table S2. Univariable analysis of association of miR-9 and miR-9* expression with achievement of complete remission and survival in AML.

Univariable analysis	HR/OR	s.e.	z	P	95% CI
miR-9					
CR	1.018	0.055	0.33	0.739	0.915- 1.133
EFS	0.983	0.025	-0.67	0.506	0.935-1.034
RFS	0.978	0.033	-0.65	0.518	0.914-1.046
OS	0.985	0.027	-0.55	0.585	0.933-1.040
miR-9*					
CR	1.159	0.102	1.68	0.092	0.976-1.376
EFS	0.889	0.039	-2.69	0.007	0.815-0.969
RFS	0.906	0.054	-1.66	0.096	0.806-1.018
OS	0.868	0.041	-3.03	0.002	0.791-0.951

Logistic regression was used for CR, and Cox proportional hazards model was used for EFS, RFS, and OS. HR < 1 or HR > 1 indicate a decreased or increased risk. OR < 1 or OR > 1 indicated a decreased or increased odds for reaching CR.

Abbreviations: AML, acute myeloid leukemia; CR, complete remission; EFS, event-free survival; RSF, relapse-free survival; OS, overall survival; HR, hazard ratio; OR, odds ratio; CI, confidence interval.

Table S3. Clinical and genetic characteristics of AML patients that express miR-9* by the level of expression. Patients were dichotomized into high and low expression groups based on the 75th percentile of miR-9* expression value (see Materials and Methods).

	Low miR-9*		High miR-9*		
	No. of cases	%	No. of cases	%	P
Clinical parameters					
Age (mean)	44.9		43.8		
Range (min-max)	17.0-60.0		18.0-60.0		
WBC, x 10^9/L (mean)	50.6		48.2		
Range (min-max)	0.8-263.4		0.8-371.0		
Sex					
Total	252		84		
Male	126	*50*	41	*49*	
Female	126	*50*	43	*51*	
ELN prognostic risk†					
Favorable	73	*29*	19	*23*	
Intermediate-I	100	*40*	33	*39*	
Intermediate-II	34	*13*	20	*24*	0.038
Adverse	45	*18*	12	*14*	
Cytogenetics					
+8	10	*4*	4	*5*	
-5 or -5q	2	*1*	0	*0*	
-7 or -7q	5	*2*	1	*1*	
-9q	3	*1*	1	*1*	
11q23‡	7	*3*	4	*5*	
t(9;11)	1	*0*	10	*12*	<0.001
t(8;21)	3	*1*	0	0	
inv(3) or t(3;3)	3	*1*	0	*0*	
inv(16)	19	*8*	2	*2*	
Normal karyotype	147	*58*	50	*60*	
Complex karyotype	27	*11*	5	*6*	
Other	25	*10*	7	*8*	
Molecular genetics in NK					
CEBPA double	2	*1*	1	*1*	
FLT3-ITD	67	*27*	19	*23*	
FLT3-TKD	9	*4*	6	*7*	
NPM1	101	*40*	27	*32*	
Treatment protocol					
HOVON04	6	*2*	1	*1*	
HOVON04A	6	*2*	0	*0*	
HOVON29	27	*11*	9	*11*	
HOVON42	48	*19*	16	*19*	
HOVON42A	69	*27*	13	*15*	0.028
HD98A	96	*38*	45	*54*	0.015

Mann-Whitney test was used for continuous variables. Fisher's exact and chi-square tests were used for categorical variables. †Only patients with biallelic mutations in *CEBPA* were included in favorable risk group (instead of any *CEBPA* gene mutation). ‡11q23 category contains *MLL*-related leukemias harboring 11q23 abnormalities other than t(9;11). NOTE. Percentages may add up >100% because of rounding.

Abbreviations: AML, acute myeloid leukemia; WBC, white blood cell count; ELN, European LeukemiaNet; NK, normal karyotype; CEBPA, CCAAT/enhancer-binding protein α; FLT3-ITD, internal tandem duplications in the FMS-like tyrosine kinase 3; FLT3-TKD, FMS-like tyrosine kinase 3 with mutations in tyrosine kinase domain; NPM1, nucleophosmin.

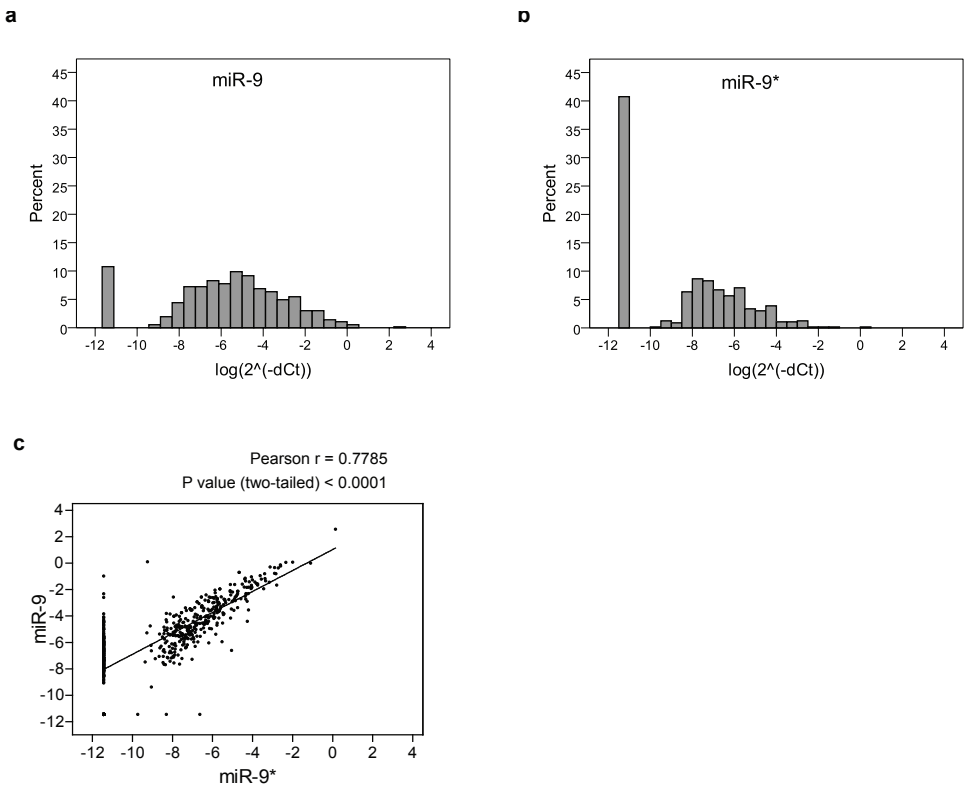

Figure S1. MiR-9 and miR-9* expression in AML. Distribution of (**a**) miR-9 and (**b**) miR-9* expression, and (**c**) the correlation between them. RNU24 was used as endogenous control. Expression is given as logarithm of $2^{(dCt)}$. For measurements below detection, the minimal threshold was set to-dCt value of-15.

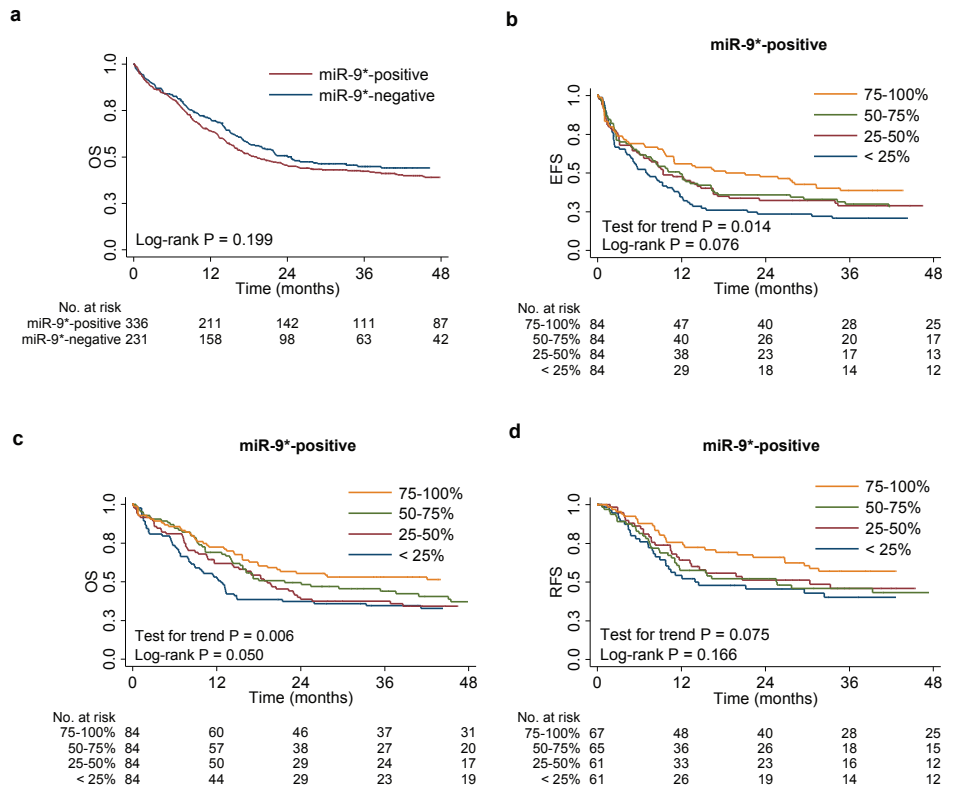

Figure S2. Patient outcome improves with increasing levels of miR-9* expression. (**a**) Overall survival (OS) of AML patients that express measurable levels of miR-9* (miR-9*-positive) and those with no expression (miR-9*-negative). (**b**) Event-free survival (EFS), (**c**) overall survival (OS), and (**d**) relapse-free survival (RFS) of miR-9*-positive cases according to the quartiles of miR-9* expression.

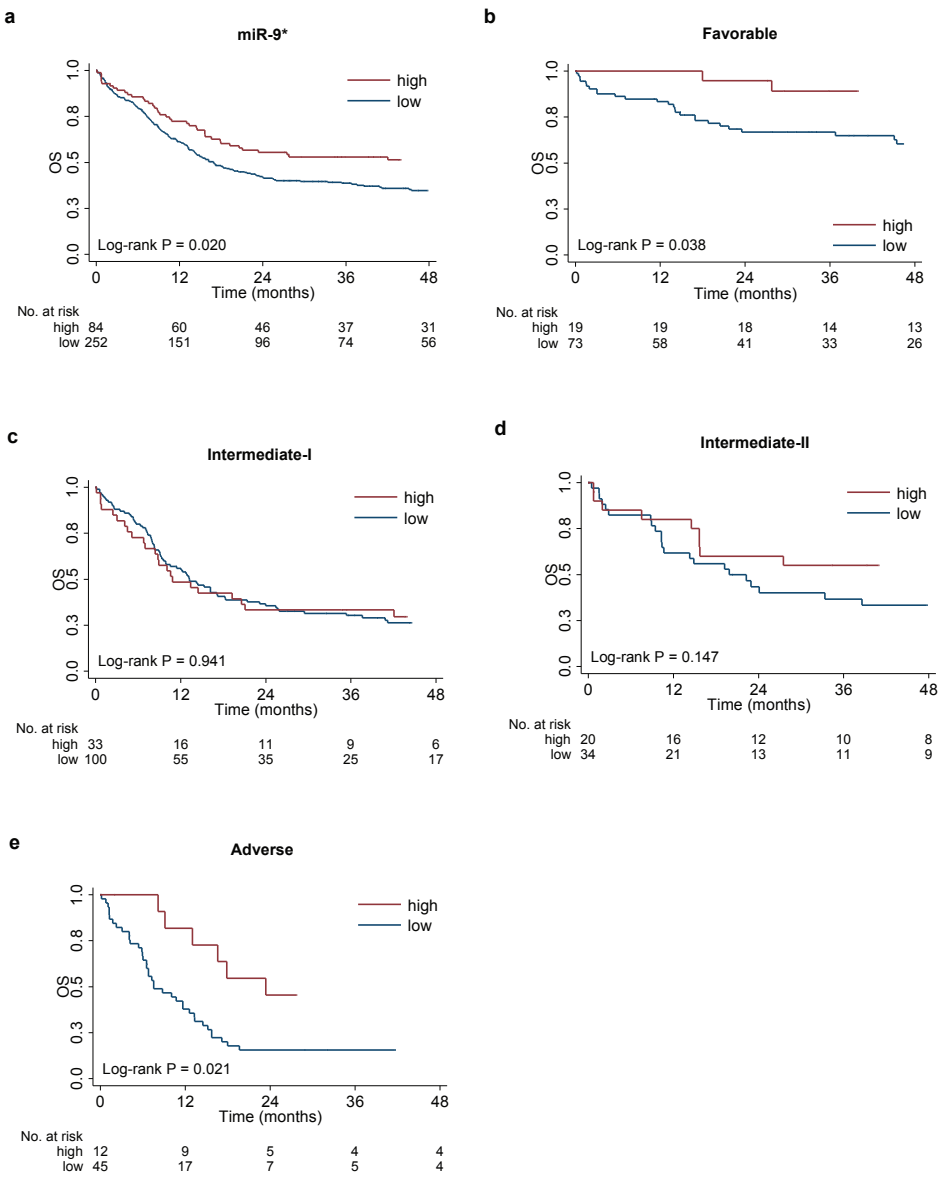

Figure S3. Patients with high miR-9* have better overall survival. (**a**) Overall survival (OS) of AML patients according to miR-9* expression. (**b-e**) OS of AML patients belonging to (**b**) favorable, (**c**) intermediate-I, (**d**) intermediate-II, and (e) adverse risk group according to miR-9* expression. Patients were dichotomized into high and low expression groups based on the 75th percentile of miR-9* expression value (see Materials and Methods).

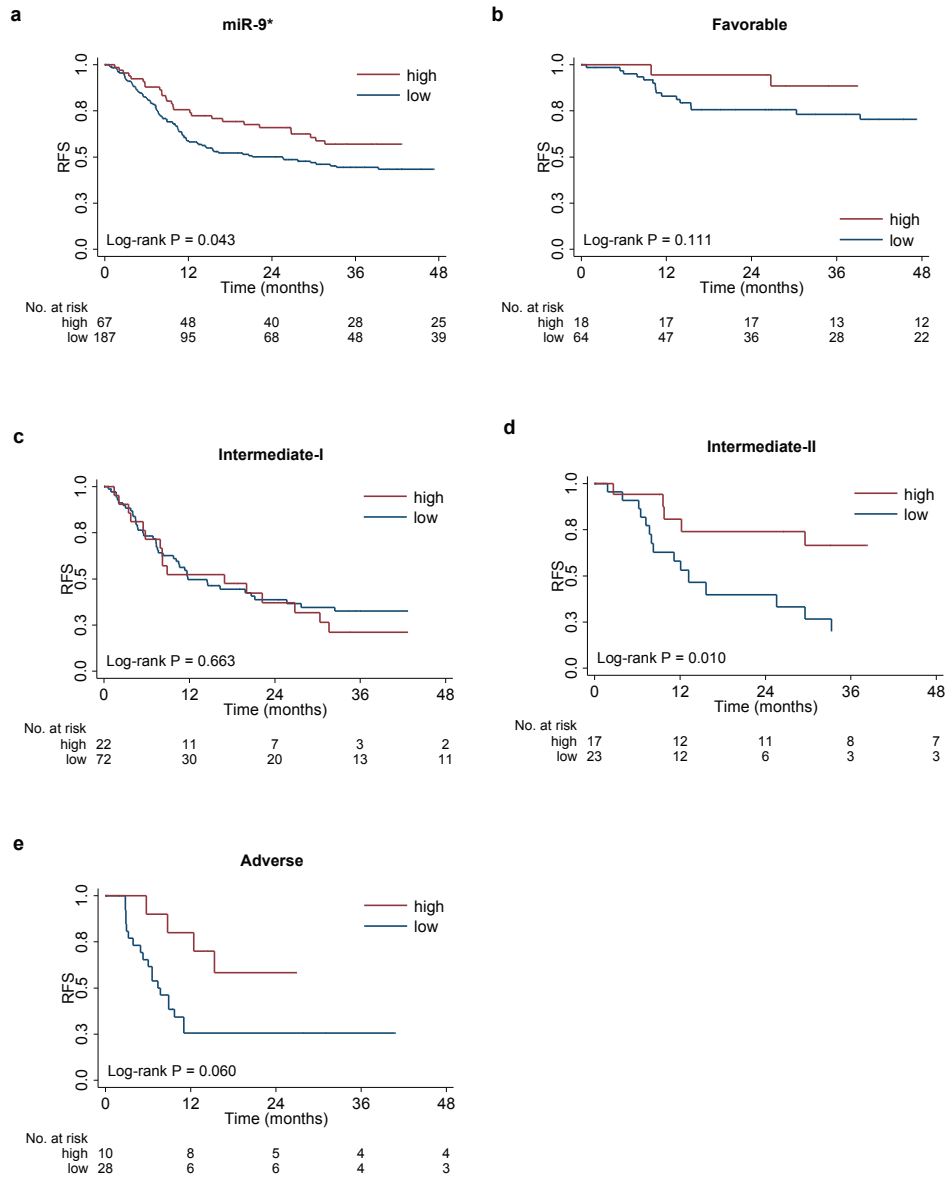

Figure S4. Patients with high miR-9* have better relapse-free survival. (**a**) Relapse-free survival (RFS) of AML patients according to miR-9* expression. (**b-e**) RFS of AML patients belonging to (**b**) favorable, (**c**) intermediate-I, (**d**) intermediate-II, and (**e**) adverse risk group according to miR-9* expression. Patients were dichotomized into high and low expression groups based on the 75th percentile of miR-9* expression value (see Materials and Methods).

6

SUMMARY AND GENERAL DISCUSSION

Partially submitted.

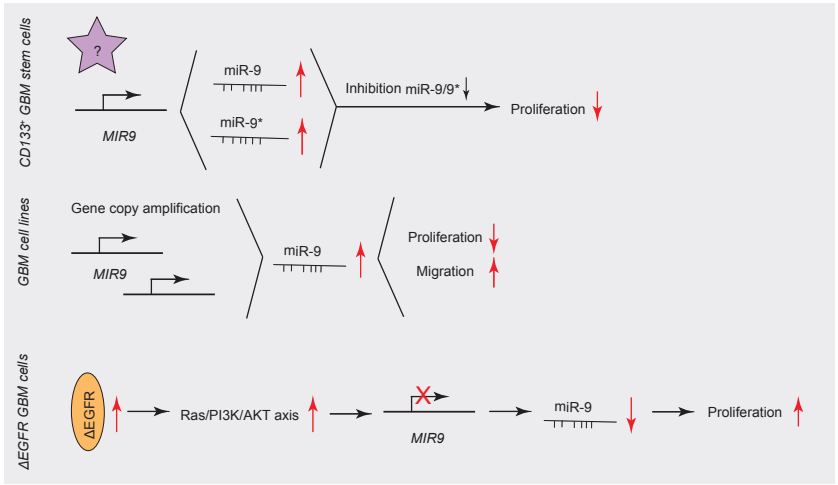

Figure 1. miR-9 and miR-9* functions in human cancer. GBM, glioblastoma multiforme; ΔEGFR, mutant epidermal growth factor receptor; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT, protein kinase B; BC, breast cancer; MEK1/2i, mitogen-activated protein kinase enzymes 1 and 2 inhibitors; SCC, squamous cell carcinoma; HPV E6, human papillomavirus E6 oncoprotein; AC, adenocarcinoma.

Here we will place the findings reported in this thesis on miR-9/9* into a broader perspective and discuss the implications for our understanding of the biology of leukemia and future research.

1. At which stage of myeloid development do miR-9/9* block myeloid differentiation and does the observed *in vitro* block in differentiation translate into aberrant hematopoiesis *in vivo*?

miR-9 has been reported to influence cell differentiation in various types of human cancer. In cervical squamous cell carcinoma, a chromosomal gain of 1q is linked with malignant progression and results in upregulation of miR-9 (1q23.3) (**Figure 1**).[1] Overexpression of miR-9 in normal keratinocytes cell line blocks epithelial differentiation. In Hodgkin lymphoma cells, miR-9 is highly expressed and its downregulation triggers B-cell differentiation into plasma cells.[2] In line with these findings, in **Chapter 2** we show that the expression of miR-9/9* disrupts normal neutrophil differentiation in the myeloid 32D cell line model and in primary HSPCs *in vitro*. In our murine miR-9/9* bone marrow overexpression model, we observed an increase in the frequency of $Lin^{-}c\text{-}Kit^{+}Sca\text{-}1^{-}$ (LK) progenitor population in cells expressing miR-9/9*. Since the frequencies of more immature $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) populations were similar, our data suggest that the observed block in differentiation may occur at the later stages of myeloid development. In order to investigate the effect of overexpression of miR-9/9* on the function of different cell populations further *in vivo* hematopoietic repopulation studies would be useful. In such experiments, HSPCs that overexpress miR-9/9* should be transplanted into recipient animals in order to evaluate their ability to repopulate different cellular compartments of the hematopoietic system in short-term and long-term repopulation experiments. Moreover, proposed transplantation experiments may help to elucidate whether our *in vitro* findings can be extrapolated into miR-9/9* induced aberrant hematopoiesis *in vivo*.

2. How do miR-9/9* influence adhesion and migration of hematopoietic cells?

In **Chapter 4**, we report that HSPCs that overexpress miR-9/9* have decreased potential to home to the BM. Data presented in **Chapter 3** revealed that miR-9/9* may regulate genes that are involved in decreased adhesion and migration of cells. Our results suggest that miR-9/9* may not only impact myeloid differentiation but also affect other aspects of HSPC function, such as adhesion, migration, engraftment and metastasis. In the past years, several studies have reported on the relationship of miR-9/9* expression with different processes in human cancer, e.g. migration and metastasis (**Table 1**, **Figure 1**). In glioblastoma multiforme, miR-9 has been reported to play a critical role in determination of the so-called “go or grow” phenotype.[3] miR-9 is part of a feedback minicircuitry that allows a tight control of the expression levels of target genes that coordinate the proliferation and migration of glioblastoma cell lines. In 2010, Ma *et al.* reported that miR-9 plays an important role in metastasis of MYC-driven breast tumor.[4] By targeting E-cadherin in

Table 1. The summary of various functions of miR-9/9* in human cancer.

	GBM				BC				CC		SCC		Hematological malignancies				
	CD133$^+$	Cell lines	ΔEGFR	Chemo-resistant	ER$^+$	TNBC	Claudin-low TNBC	Metastatic	CSCC	CA	Skin	Oral	ALL	HL	MM	WM	AML
Proliferation	9a	3,33	34				10a		1	35		36,37	38a			39b	19,20,40,41
Differentiation	9								1,42					2,43			19
Migration		3				44,45	10b	4,46,47	1	35							
Chemo/drug resistance				48a	49										50		
Self-renewal				51b													20
Tumorigenicity				51b						35							
Invasion						44,45	10b	4,46,47			52						
Metastasis								4,47			52						
Increased cell frequency											52						
Apoptosis																39b	20,41
Autophagy																39b	

Numbers represent the reference number of the publication where the given function has been reported.
a: Function is attributed to both miR-9 and miR-9*.
b: Function is attributed to miR-9* only.
Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BC, breast cancer; CA, cervical adenocarcinoma; CC, cervical cancer; CSCC, cervical squamous cell carcinoma; ΔEGFR, mutant epidermal growth factor receptor; ER, estrogen receptor; GBM, glioblastoma multiforme; HL, Hodgkin lymphoma; MM, multiple myeloma; SCC, squamous cell carcinoma; TNBC, triple-negative breast cancer; WM, Waldenström macroglobulinemia

breast tumor cells, miR-9 enables dissociation of carcinoma cells and leads to formation of pulmonary micrometastasis.[4,5] Retention and movement of HSPCs within the BM and periphery are important biologic processes that are crucial to the maintenance of the stem cell pool in the BM.[6,7] The detailed ability of miR-9/9* to influence adhesion and migration of HSPCs warrants further study for example using *in vitro* assays that measure adhesion to fibronectin and migration of HSPCs towards various ligands (e.g. CXCL2).[8]

3. Do miR-9 and miR-9* exert synergistic or antagonistic functions?

The data presented in this thesis are derived from experiments with concomitant overexpression of both mature miRNA strands, miR-9 and miR-9*. This implies that we are unable to distinguish the effects of either one separately and therefore we cannot make specific interpretations regarding their independent roles nor about potentially synergistic or antagonistic roles of miR-9 and miR-9*. In CD133$^+$ glioblastoma stem cells, both miRNAs are highly expressed and needed for stem cell renewal (**Figure 1**).[9] In claudin-low triple-negative breast cancer cells, overexpression of miR-9* has been reported to synergize with mitogen-activated protein kinase enzymes 1 and 2 (MEK1/2) inhibitor and to suppress growth, migration and invasion of tumor cells.[10] Interestingly, miR-9 has a reverse effect and its overexpression together with MEK1/2 inhibitor increases cell proliferation. These findings suggest that miRNAs co-expressed following generation from the same precursor may fulfill different and opposing functions and that there may be regulatory mechanisms specific of miR-9 or miR-9* expression. In **Chapter 2**, we show that miR-9 and miR-9* are both highly expressed in most cases of AML. The functions of individual miR-9 and miR-9* in hematopoietic system remain to be studied using models with specific overexpression and/or silencing of either one of these miRNAs.

4. Which targets of miR-9/9* contribute to their functions observed in myeloid cells?

In **Chapter 2**, using GEP assay we identified ERG as a miR-9 target in murine 32D cells and human AML samples. Reintroduction of Erg into miR-9/9* overexpressing 32D cells restored normal neutrophil differentiation. The proteomics data presented in **Chapter 3** indicate other potential miR-9 targets that may influence differentiation, adhesion and migration of myeloid cells. These include MYH9, MYO1C, ITGA6, VCL and ANXA2. Until today, 4 of them have been reported in relation to miR-9 in different types of human cancer. *MYO1C*, *ITGA6* and *VCL* are repressed by miR-9 in osteosarcoma, and *ANXA2* in hepatocellular carcinoma.[11,12] Functions of these potential targets have been linked to the hematopoietic system. MYO1C and VCL are involved in cytoskeleton rearrangements and they may influence cell motility in B cells and monocytes, respectively.[13,14] ITGA 6 is associated with resistance of leukemia and lymphoma cells against gamma T cell-mediated cytotoxicity and ANXA2 may affect adhesion, homing and chemosensitivity of acute lymphoblastic leukemia cells.[15,16] Whether the proteins reported in **Chapter 3** are direct targets of miR-9 and influence normal myeloid cell functions needs to be further investigated. miRNAs

fine-tune cell-specific gene regulatory networks by influencing the expression of multiple genes. These genes may interplay with each other by fulfilling separate functions. Therefore, deconstructing the weighted contribution of each of these separate targets to the observed phenotypes may be a challenging task.

5. What are the mechanisms that regulate miR-9/9* expression in AML?

The reasons for aberrant expression of miR-9/9* in AML are unknown. Since miR-9/9* genes contain their own promoters, one could speculate that it is a result of deregulated control by aberrant levels of transcription factors that regulate miR-9/9* expression. In fact, miR-9/9* expression has been reported to be regulated by transcription factors commonly upregulated in AML, such as Nf-κB.[17,18] The mechanisms that control miR-9/9* levels may be different in various subtypes of AML since it has been shown that various aberrantly expressed fusion proteins can influence miR-9 expression. In example, the leukemic fusion protein AML1-ETO downregulates miR-9 in t(8;21) rearranged leukemias and MLL-AF9 upregulates miR-9 in *MLL*-rearranged cases.[19,20] The existence of various mechanisms that may regulate miR-9/9* within one malignancy has been shown in other types of human cancer where miR-9/9* are differentially expressed according to the cellular context (**Figure 1**).

In **Chapters 2**, we reported that miR-9 is expressed at low levels in normal $CD34^+$ cells, while miR-9* is not expressed. Furthermore, miR-9 is expressed in 89% and miR-9* in 59% of patients with AML as shown in **Chapter 5**. The molecular and mechanistic basis of low miR-9* expression in such a high percentage of AML cases remains the subject of future studies. The existence of specific post-transcriptional modulation mechanisms that regulate miR-9* expression separately from miR-9 may play a role, e.g. RNA methylation and differential expression of RNA-induced silencing complex components.[21-23]

6. Can the prognostic value of miR-9* expression be incorporated in algorithms with clinical utility that predict patient outcome in AML?

Few miRNAs have been shown to impact patient outcome in AML, e.g. miR-155, miR-181a and miR-212.[24-28] In **Chapter 5**, we show that although miR-9 has no prognostic significance, its passenger strand miRNA-9* predicts improved survival. Until today, it is the first report showing that the preservation of a miRNA* expression has prognostic significance. Most miRNA*s are thought to be degraded during the strand selection by miRISC complexes.[29,30] Therefore, our results support further research focused on miRNA*s in AML.

The use of miRNAs as biomarkers of a disease is still hindered by technical obstacles and no miRNA has yet been incorporated into clinical algorithms. Before we can apply miRNAs in the clinical setting, the measurement of their expression levels should be standardized and robust.[31] Additionally, the prognostic value of miRNAs is usually reported for “high versus low” expressers, unlike “yes versus no” for gene mutations, e.g. NPM1 and C/EBPα.[32] This brings difficulties in establishing universal cut-offs of miRNA expression that would be clinically applicable.

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A

ADDENDUM

ENGLISH SUMMARY

miRNAs are small non-coding RNAs that as a part of miRISC complexes bind to the target mRNAs and lead to translational repression and/or transcript decay. In this way, they post-transcriptionally suppress gene expression. miRNAs are known to control the expression of genes involved in normal myelopoiesis and AML, a disease that is characterized by the accumulation of abnormally differentiated myeloid cells in the BM.

In **Chapter 2**, a screen using retroviral barcoded miRNA expression library in murine 32D cell line model revealed that miR-9/9* may interfere with normal neutrophil differentiation. This was further confirmed by the overexpression of miR-9/9* in 32D cells and in murine primary HSPCs. In human $CD34^+$ cells, miR-9 is expressed at low levels and miR-9* is not expressed. However, they are both aberrantly upregulated in most cases of AML. In order to identify the miR-9/9* targets that may be involved in the observed phenotype, we analyzed the transcriptomes of AML cells that highly express miR-9/9* and 32D cells that were transduced with these miRNAs. We found ERG to be the only potential target common for both sample types. ERG was further shown to be direct miR-9 target and its overexpression in 32D cells recued miR-9/9*-induced block in neutrophil differentiation.

To unravel other potential miR-9/9* targets that may be involved in normal myeloid cell function, in **Chapter 3** we performed proteome studies in 32D cells that ectopically expressed miR-9/9*. Twenty-nine proteins were significantly down- or upregulated in a steady state between miR-9/9* transduced and control cells, and 42 upon induction of neutrophil differentiation. Subsequent pathway analysis showed that these proteins could be related to cell differentiation, apoptosis, migration and adhesion. Among the downregulated proteins, we found 5 potential miR-9 targets: MYO1C, ANXA2, VCL, MYH9 and ITGA6. No miR-9* targets were found to be differentially expressed.

In order to explore potential influence of miR-9/9* on migration and adhesion of normal hematopoietic cells, in **Chapter 4** we examined the function of HSPCs that ectopically expressed miR-9/9* using *in vivo* homing assay. We observed that HSPCs that were transduced with miR-9/9* seed in the BM three times less efficiently than control cells. The observed phenotype was not related to the increased apoptosis and/or decreased colony-forming potential of these cells. These preliminary results suggest that miR-9/9* may influence adhesion and migration of HSPCs.

The differential expression of miRNAs has been associated with various subtypes of AML and linked with clinical outcome. In **Chapter 5**, we investigated the prognostic significance of miR-9/9* in a large cohort of primary AML cases. Although, the expression of miR-9 had no clinical impact, high expression of its passenger strand miR-9* independently predicted improved patient survival. Furthermore, transcriptome analysis revealed that miR-9* may regulate genes involved in leukemogenesis, such as *MN1* and *MLLT3*.

DUTCH SUMMARY (NEDERLANDSE SAMENVATTING)

MiRNAs zijn kleine niet-eiwit coderende RNA's die als onderdeel van het miRISC complex binden aan target mRNAs, wat resulteert in translatie onderdrukking en of transcriptie blokkade. miRNAs reguleren de expressie van genen die betrokken zijn bij de normale myelopoiese en bij AML, een ziekte die wordt gekenmerkt door de accumulatie van abnormaal gedifferentieerde myeloïde cellen in het beenmerg.

In **Hoofdstuk 2** met behulp van een retrovirale barcode-miRNA expression-library in een muizen 32D-cellijn model bleek dat miR-9/9* kunnen interfereren met de normale neutrofiele differentiatie. Dit werd bevestigd door overexpressie van miR-9/9* in 32D-cellen en in primaire humane pluripotente stamcellen (HSPCs). In humane $CD34^+$ cellen, komt miR-9 in geringe mate en miR-9 * niet tot expressie. Echter, beiden komen tot expressie in de meerderheid van AML patiënten. Om de miR-9/9* targets die betrokken zijn bij het waargenomen fenotype te kunnen identificeren, analyseerden we het transcriptoom van AML cellen en 32D cellen die waren getransduceerd met deze miR-9/9* en identificeerden we ERG als een potentieel target. Vervolgens hebben we aangetoond dat ERG een directe miR-9 target is en dat over-expressie van ERG in 32D cellen een miR-9/9*-geïnduceerde blok in neutrofielen differentiatie kan herstellen.

Om nieuwe potentiële miR-9/9* targets die betrokken zijn bij normale myeloïde celfunctie te kunnen identificeren, in **Hoofdstuk 3** voerden we proteomics-studies uit in 32D cellen met ectopisch expressie van miR-9/9*. In de steady state toestand waren 29 eiwitten significant up- of down-gereguleerd in miR-9/9* cellen te opzichte van controle cellen en bij in-vitro inductie van neutrofielen differentiatie waren dat er 42. Een daaropvolgende pathway analyse toonde aan dat deze eiwitten te maken hebben met celdifferentiatie, apoptose, migratie en adhesie. Onder de down-gereguleerde eiwitten vonden we 5 mogelijke miR-9 targets: MYO1C, ANXA2, VCL, MYH9 en ITGA6. miR-9 * targets bleken niet verschillend tot expressie te komen.

Met behulp van een *in vivo* homing assay in **Hoofdstuk 4** hebben we de potentiele invloed van miR-9/9* op de migratie en adhesie van normale hematopoietische cellen onderzocht in HSPCs met ectopische expressie van miR-9/9* We vonden dat HSPCs die waren getransduceerd met miR-9/9* driemaal minder efficiënt konden nestelen in het beenmerg dan controle cellen. Het waargenomen fenotype was niet gerelateerd aan verhoogde apoptose of aan een verminderd kolonievormend vermogen van deze cellen. Deze voorlopige resultaten suggereren dat miR-9/9 * de adhesie en migratie van HSPCs kunnen beïnvloeden.

Tenslotte in **Hoofdstuk 5** hebben we beschreven dat de differentiële expressie van miRNAs is geassocieerd met verschillende subtypes van AML en gekoppeld aan de klinische uitkomst. We onderzochten de prognostische betekenis van miR-9/9* in een groot cohort van primaire AML casus. Hoewel, de expressie van miR-9 niet was geassocieerd met de uitkomst, voorspelde een hoge expressie van miR-9* een verbeterde overleving van patiënten.

POLISH SUMMARY (POLSKIE PODSUMOWANIE)

miRNA to małe niekodujące RNA, które jako część kompleksów miRISC wiążą się z docelowymi mRNA i blokują ich translację oraz/albo prowadzą do rozpadu docelowych transkryptów. W ten sposób potranslacyjnie obniżają ekspresję genów. Wiadomo, że miRNA kontrolują ekspresję genów zaangażowanych w leukopoezę i ostrą białaczke szpiku, chorobę charakteryzującą się nowotworowym rozrostem w szpiku wadliwie zróżnicowanych komórek krwi.

W **Rozdziale 2** skrining z użyciem retrowirusowej oznakowanej biblioteki miRNA w mysiej linii komórkowej 32D ujwnił, że miR-9/9* mogą interferować z normalnym różnicowaniem się neutrofilów. To zostało dalej potwierdzone przez nadekspresję miR-9/9* w komórkach 32D i w mysich pierwotnych komórkach macierzystych i prekursorowych. W ludzkich normalnych komórkach macierzystch krwi ($CD34^+$) miR-9 ulega ekspresji na niskim poziomie i miR-9* nie ulega ekspresji. Jednakże oba miRNA występują na anormalnie wysokim poziomie w większości przypadków ostrej białaczki szpiku. Aby zidentyfikowć docelowe dla miR-9/9* transkrypty, które mogą być zaangażowane w zaobserwowany fenotyp przeanalizowaliśmy transkryptomy komórek ostrej białaczki szpiku, które charakteryzowały się wysokim poziomem miR-9/9* oraz transkryptomy komórek 32D, w których miR-9/9* były ekpresjonowane za pomocą transdukcji. Odkryliśmy, że ERG był jedynym potencjalnym kandydatem w obu typach komórek. Białko ERG zostało dalej potwierdzone jako bezpośredni cel regulwany przez miR-9 i jego nadekspresja w komórkach 32D odwróciła spowodowaną przez miR-9/9* blokadę w różnicowaniu się neutrofilów.

Aby ujawnić inne potencjalne cele miR-9/9*, które mogą być zaangażowane w normalne funkcjonowanie leukocytów w **Rozdziale 3** przeprowadziliśmy badania nad proteomem w komórkach 32D z użyciem ektopowej ekspresji miR-9/9*. W normalnym stanie 29 białek było ekspresjonowanych na znacząco niższym lub wyższym poziomie w komórkach transdukowanych miR-9/9* w porównaniu z komórkami kontrolnymi oraz 42 białka były ekspresjonowane na anormalnym poziomie podczas indukcji różnicowania się neutrofilów. Późniejsza analiza wykazała, że te białka mogą być związane z różnicowaniem się komórek, apoptozą, migracją i adhezją. Wśród białek, które ulegały ekspresji na zredukowanym poziomie odkryliśmy 5 potencjalnych celów dla miR-9: MYO1C, ANXA2, VCL, MYH9 and ITGA6. Nie odkryliśmy żadnych potencjalnych celów dla miR-9*.

W celu zbadania czy miR-9/9* mogą potencjalnie wpływać na migrację i adhezję normalnych komórek krwi, w **Rozdziale 4** zbadaliśmy funkcję komórek macierzystych i prekursorowych, które ektopowo ekspresjonowały te miRNA, z użyciem badania *in vivo* zasiedlania szpiku po przeszczepie. Zaobserwowaliśmy, że komórki macierzyste i prekursorowe, które ekspresjonowały miR-9/9* zasiedlają szpik trzy razy mniej wydajnie niż komórki kontrolne. Zaobserwowany fenotyp nie był związany ze zwiększoną apoptozą oraz/albo zmniejszonym potencjałem do tworzenia kolonii. Te wstępne wyniki sugerują, że miR-9/9* mogą mieć wpływ na adhezję i migrację komórek macierzystych i prekursorowych krwi.

Zróżnicowana ekspresja miRNA jest związana z różnymi rodzajami ostrej białaczki szpiku i wskaźnikiem przeżywalności pacjentów. W **Rozdziale 5** badaliśmy prognostyczne znaczenie miR-9/9* w dużej kohorcie pierwotnych przypadków ostrej białaczki szpiku. Mimo, że ekspresja miR-9 nie miała znaczenia klinicznego, wysoka ekspresja miR-9* była niezależnie związana z lepszą przewidywaną przeżywalnością pacjentów. Ponadto analiza transkryptomu ujawniła, że miR-9* może regulować geny zaangażowane w rozwój białaczki, takie jak *MN1* i *MLLT3*.

GLOSSARY OF ABBREVIATIONS

(in alphabetic order)

AC	adenocarcinoma
AKT	protein kinase B
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
AMLSG	German-Austrian AML Study Group
AP-1	activator protein 1
APL	acute promyelocytic leukemia
BC	breast cancer
BM	bone marrow
CBFβ	core binding factor β
CDK6	cyclin-dependent kinase 1
C/EBPα	CCAAT/enhancer-binding protein α
C/EBPβ	CCAAT/enhancer-binding protein β
CFU	colony-forming unit
CFU-G	CFU-granulocyte assay
CI	confidence interval
CLL	chronic lymphocytic leukemia
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CR	complete remission
DC	dendtritic cell
ΔEGFR	mutant epidermal growth factor receptor
DFS	disease-free survival
EFS	event-free survival
ELN	European LeukemiaNet
ERG	ETS-related gene
ErP	erythrocyte precursor
ERRFI1	ERBB receptor feedback inhibitor 1
EV	empty vector
FACS	fluorescence-activated cell sorting
FDR	false discovery rate
FGFR1	fibroblast growth factor receptor 1
FZD7	frizzled-7
GBM	glioblastoma multiforme
G-CSF	granulocyte colony-stimulating factor

GEP	gene expression profile
GMP	granulocyte/monocyte progenitor
HCC	hepatocellular carcinoma
HDAC	histone deacetylase
HES1	hairy and enhancer of split-1
HL	Hodgkin lymphoma
HOVON	Dutch-Belgian-Hematology-Oncology-Cooperative group
HPV E6	human papillomavirus E6 oncoprotein
HR	hazard ratio
HSC	hematopoietic stem cell
HSPC	hematopoietic stem/progenitor cell
IGF2BP3	insulin-like growth factor 2 mRNA binding protein 3
IL-3	interleukin 3
IPA	Ingenuity Pathway Analysis
LC-MS/MS	liquid chromatography tandem mass spectrometry
Lin^-	lineage negative cells
LK	$Lin^-c\text{-}Kit^+Sca\text{-}1^-$
LNA	locked nucleic acid
LSK	$Lin^-Sca\text{-}1^+c\text{-}Kit^+$
LSC	leukemia stem cell
LSPC	leukemic stem/progenitor cell
MEF2C	myocyte-specific enhancer factor 2C
MEK1/2(i)	mitogen-activated protein kinase enzymes 1 and 2 (inhibitors)
MEP	megakaryocyte/erythroid progenitor
miRISC	miRNA-induced silencing complex
miR(NA)	microRNA
miR-9/9*	miR-9 and miR-9*
MkP	megakaryocyte precursor
MLL	mixed-lineage leukemia
MM	multiple myeloma
MNC	mononuclear cells
MPP	multipotent progenitor
muBM	murine bone marrow cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NF1A	nuclear factor 1 A-type
NK	natural killer cell
NK-AML	normal karyotype AML
NPM1	nucleophosmin gene
OR	odds ratio
OS	overall survival
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PRDM1/BLIMP-1	positive regulatory domain 1
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PKB/AKT1	protein kinase B
RFS	relapse-free survival
RHOH	Ras homolog family member H
RNA	ribonucleic acid
RUNX1	runt-related transcription factor 1
RYBH	YY1-binding protein
SCC	squamous cell carcinoma
SILAC	stable isotope labeling by amino acids in cell culture
SOCS1	suppressor of cytokine signaling 1
SPRED1	sprouty-related, EVH1 domain-containing protein 1
STAT3	signal transducer and activator of transcription 3
TNF-α	tumor necrosis factor α
WBC	white blood cells
WM	Waldenström macroglobulinemia
TCGA	The Cancer Genome Atlas
3'UTR	3' untranslated region

CURRICULUM VITAE

Katarzyna Nowek was born in Zgorzelec, Poland, in 1986. After receiving her high school diploma in foreign languages from Liceum Ogólnokształcące im. Braci Śniadeckich (Zgorzelec, Poland), she studied Biotechnology with the specialization in Molecular Biotechnology and Biocatalysis at the Wrocław University of Technology. After three-year research internship at the Biochemistry Department when she undertook genetic engineering in order to examine changes in amino acid microenvironment, she successfully defended her thesis 'Single-point mutation screening of the Trp residues microenvironment in the intrinsically disordered protein Starmaker' and graduated in Wrocław in 2010. In October 2010 she moved to the Netherlands and she was appointed a PhD candidate at the Department of Hematology at Erasmus Medical Center in Rotterdam. Here, she studied the role of miR-9/9* in myeloid development and acute myeloid leukemia.

LIST OF PUBLICATIONS

Nowek K, Sun SM, Bullinger L, Bindels EM, Exalto C, Dijkstra MK, van Lom K, Döhner H, Erkeland SJ, Löwenberg B, Jongen-Lavrencic M. Aberrant expression of miR-9/9* in myeloid progenitors inhibits neutrophil differentiation by post-transcriptional regulation of ERG. *Leukemia*. 2016 Jan; 30(1):229-37. doi: 10.1038/leu.2015.183.

Nowek K, Sun SM, Dijkstra MK, Bullinger L, Döhner H, Erkeland SJ, Löwenberg B, Jongen-Lavrencic M. Expression of a passenger miR-9* predicts favorable outcome in adults with acute myeloid leukemia less than 60 years of age. *Leukemia*. 2016 Feb; 30(2):303-9. doi: 10.1038/leu.2015.282.

Nowek K, Wiemer EA, Jongen-Lavrencic M. The versatile nature of miR-9/9* in human cancer. *Submitted*

Hatzl S, Geiger O, Kuepper MK, Caraffini V, Seime T, Furlan T, Nussbaumer E, Wieser R, Pichler M, Scheideler M, **Nowek K**, Jongen-Lavrencic M, Quehenberger F, Wölfler A, Troppmair J, Sill H, Zebisch A. Increased Expression of miR-23a Mediates a Loss of Expression in the RAF Kinase Inhibitor Protein RKIP. *Cancer Res*. 2016 Jun 15; 76(12):3644-54. doi: 10.1158/0008-5472.CAN-15-3049.

PHD PORTFOLIO

Name PhD student:	K. Nowek
Promotors:	Prof. Dr. B. Löwenberg, Prof. Dr. R. Delwel
Co-promotor:	Dr. M. Jongen-Lavrencic
Erasmus MC department:	Hematology
Research school:	Molecular medicine
PhD period:	Oct 2010 – April 2015

PhD training	Year	Workload (Hours/ECTS)
General courses		
Course on Molecular Medicine	2011	0.7
Laboratory Animal Science (Art.9)	2011	4.2
Get-out-of-your-lab Days	2011	0.6
Biomedical English Writing Course	2014	3
Research Management for PhD-students	2014	1
In-depth courses and workshops		
Basic Introduction Course on SPSS	2011	0.6
Workshop Basic Data Analysis on Gene Expression Arrays	2011	0.7
Photoshop and Illustrator CS5 Workshop	2011	0.3
Workshop on Microsoft Excel 2010: Basic	2014	0.3
Workshop on Microsoft Excel 2010: Advanced	2014	0.4
Workshop on Microsoft Access 2010: Basic	2014	0.3
Ensemble Workshop IX	2015	0.6
Scientific meetings Department of Hematology		
Work discussions	2010-2015	10
Erasmus Hematology Lectures	2010-2015	2.5
PhD lunch with seminar speaker	2010-2015	2.5
AIO/Postdoc meeting	2010-2015	2.5
Literature discussion	2010-2015	7.5
AIO/Postdoc Career Event	2015	0.1
Inter(national) conferences		
Dutch Hematology Congress (2X)	2011, 2012	1.2
MolMed Day (2X)	2013, 2015	0.6
Annual Conference American Society of Hematology	2014	1
Daniel den Hoed Symposium	2014	1
Molecular Aspects of Hematological Disorders (2X)	2014, 2015	2
European Hematology Association Congress	2015	1
Presentations		
AIO/Postdoc meeting (oral, 4X)	2011-2014	2
Journal Club (oral, 4X)	2011-2014	2
Workdiscussion (oral, 9X)	2011-2014	4.5
Extracurricular courses		
BioBusiness Summer School	2014	
Patient Oriented Research	2015	
	Total	**46**

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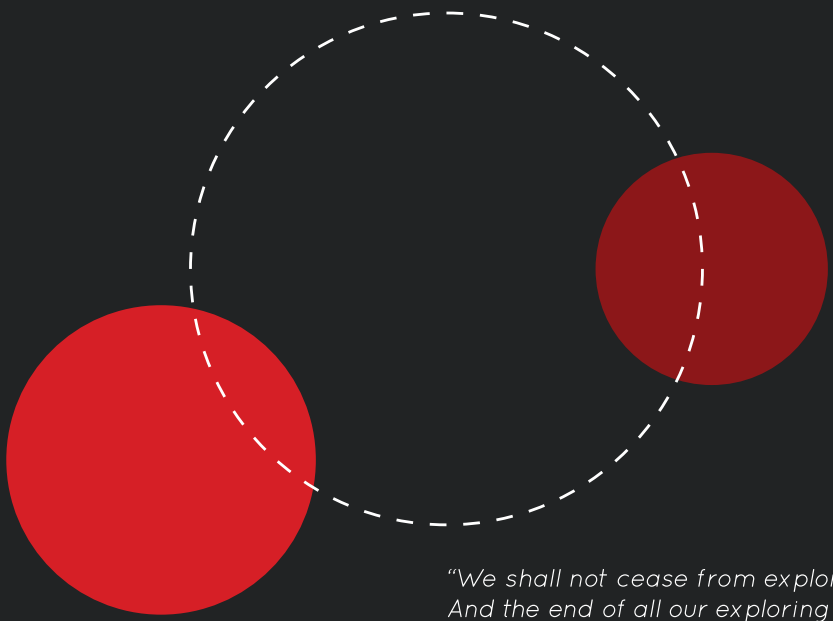
Katarzyna Nowek
Rotterdam, 23 May 2017



"We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time."

T.S. Eliot, Little Gidding