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miR-10a overexpression is associated with NPM1 mutations and MDM4 downregulation in intermediate-risk acute myeloid leukemia

Dmitriy Ovcharenko[a,b,*], Friedrich Stölzel[c,*], David Poitz[d], Fernando Fierro[c], Markus Schaich[c], Andreas Neubauer[e], Kevin Kelnar[a], Timothy Davison[a], Carsten Müller-Tidow[f], Christian Thiede[c], Martin Bornhäuser[c], Gerhard Ehninger[c], David Brown[a], and Thomas Illmer[c]

[a]*Asuragen Inc., Austin, Tx., USA;* [b]*Altogen Labs, Austin, Tex., USA;* [c]*Medizinische Klinik und Poliklinik I, Dresden University of Technology, Dresden, Germany;* [d]*Klinik für Kardiologie, Dresden University of Technology, Dresden, Germany;* [e]*Klinik für Innere Medizin, Hämatologie, Onkologie, und Immunologie, University Hospital, Giessen and Marburg und Philipps University, Marburg, Germany;* [f]*Klinik für Innere Medizin, Hämatologie und Onkologie, University of Münster, Germany*

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Objective. **The study investigated differential microRNA (miRNA) expression patterns in acute myeloid leukemia (AML) patients with intermediate-risk (IR) characteristics. After characterization and validation of *miR-10a*, which was specifically upregulated in nucleophosmin 1 (*NPM1*) mutant AML samples, functional consequences of *miR-10a* overexpression were further delineated in vitro.**

Materials and Methods. **Microarray analysis of miRNAs in bone marrow samples from AML (IR) patients with *NPM1* mutations and healthy donors was performed to detect differential expression patterns. After validation of miRNA expression specific for *NPM1* mutation in AML patients by quantitative reverse transcription polymerase chain reaction, a functional target gene search was conducted using complementary DNA microarray data from samples transfected with *miR-10a*. Potential target gene validation was done using transient transfection of K562 cells followed by Western blotting and luciferase reporter assay.**

Results. **In comparison with wild-type samples, *NPM1* mutant AML samples were shown to markedly overexpress *miR-10a*. Subsequent in vitro *miR-10a* overexpression induced differential gene expression as determined by microarray analysis. Here the murine double minute 4 (*MDM4*) gene turned out as a candidate gene for *miR-10a*. Validation of *MDM4* in leukemic cells revealed a robust negative relationship between *miR-10a* overexpression and *MDM4* downregulation. Furthermore, we determined an inverse association between *miR-10a* and *MDM4* expression in AML (IR) samples with respect to their *NPM1* mutational status.**

Conclusions. ***miR-10a*** **expression is highly characteristic for AML (IR) patients with *NPM1* mutations and may influence its biological properties in AML by interfering with the *p53* machinery partly regulated by *MDM4*.** **© 2011 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Acute myeloid leukemia (AML) is a heterogeneous disease of hematopoietic stem cells and patients are often classified according to the presence of specific cytogenetic alterations [1]. There are substantial differences in responses to treatments for various types of AML. Particular chromosomal aberrations that are frequently observed in AML blasts have been associated with treatment failure or relapse, such that the development of individualized treatment strategies tailored to patients presenting with specific genetic alterations is critical [2]. Historically, patients with normal karyotype have been defined as having intermediate-risk (IR) AML, although the course of disease progression in these individuals is highly variable [3]. Because of recent progress in the identification of genetic alterations associated with AML, the IR

*Drs. Ovcharenko and Stölzel contributed equally to this work.

Offprint requests to: Friedrich Stölzel, M.D., Medizinische Klinik und Poliklinik I, Universitätsklinikum Carl Gustav Carus der Technischen Universität, Fetscherstrasse 74, 01307 Dresden, Germany; E-mail: friedrich.stoelzel@uniklinikum-dresden.de

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designation is now further classified according to the presence of mutations in specific genes, such as those encoding Fms-like tyrosine kinase 3 (*FLT3*) [4], CCAAT/enhancer binding protein-alpha (*CEBP/α*) [5], or nucleophosmin 1 (*NPM1*) [6]. Consequently, it is known that internal tandem duplications (ITD) of *FLT3* are associated with a poor prognosis [7–9], whereas *NPM1* mutations predict a positive response to chemotherapy [10–12]. Altered FLT3 signaling is both specific and vital for the growth and survival of leukemic cells because inhibition of FLT3 induces apoptosis in *FLT3*-ITD−positive cell lines [11,13,14]. Although direct inhibition of NPM1 signaling has not been achieved, a number of studies have demonstrated the ability of mutated *NPM1* to counteract apoptosis, enhance self-renewal, and inhibit the differentiation of leukemic cells [15,16]. Moreover, *NPM1* mutations are associated with specific changes in the overall gene expression profile of myeloid cells [17,18], similar to that observed for *FLT3*-ITD mutations [19,20].

MicroRNAs (miRNAs) are small noncoding RNAs with cell-type−specific expression patterns [21]. They regulate expression of at least 5000 genes and represent one-third of the human genome [22]. miRNAs bind to partially or completely homologous sequences within the 3′ untranslated region (3′ UTR) of their mRNA targets and repress their expression at the post-transcriptional level (for review see [23]).

miRNAs are key players in genetic programs that control differentiation and embryogenesis, primarily by regulating expression of gene clusters that ultimately modulate cellular functions [24,25]. For example, overexpression of certain miRNAs is capable of driving the development of stem cells into lymphocytic and myeloid differentiation [26–28]. Similarly, changes in miRNA expression in response to the differentiating agent retinoic acid provides further evidence for the ability of miRNAs to regulate differentiation and proliferation of hematopoietic cells [29]. Interestingly, AML and acute lymphoblastic leukemia (ALL) patients can be discriminated by the expression profiles of miRNAs, which could prove to be more sensitive than screening with complementary DNA microarrays [29–31].

It is currently accepted that chromosomal abnormalities critically influence miRNA expression levels. For example, chromosomal translocation deregulates *miR-142* expression in t(8;17)-positive prolymphocytic leukemia [32] and *miR-223* expression in t(8;21)-positive AML [33]. Furthermore, *miR-10a* overexpression in *NPM1* mutant AML samples has recently been described [34]. Because the majority of AML patients do not have karyotypic alterations, we investigated AML patients with IR characteristics for specific alterations in miRNA expression in order to delineate the molecular consequences of these alterations.

Materials and methods

Patient characteristics and cell isolation

Bone marrow (BM) blast samples of AML patients with IR characteristics, included in the prospective AML96 and AML2003 trials of the Study Alliance Leukemia, were obtained at diagnosis by routine BM aspiration. The studies were approved by the institutional review board of the Medical Faculty of the Technical University, Dresden, Germany.

Mononuclear cells were isolated by density gradient centrifugation using Ficoll-Hypaque (1.077 g/mL) and cryopreserved in aliquots containing 5 to 20 × 10^6 cells/sample. Normal BM samples were acquired from healthy donors, and mononuclear cells were prepared and cryopreserved. All donors gave written informed consent for the use of their samples, and all patients were homogenously treated as described previously. Unmodified BM samples were further used for miRNA isolation as described for the microarray experiments. For quantitative reverse transcription polymerase chain reaction (qRT-PCR) experiments BM samples of healthy control donors were further processed. Isolation of $CD34^+$ cells, $CD3^+$ T cells and granulocytes was done as described previously [35].

AML risk classification

Chromosome analyses were performed as described previously [36]. The AML (IR) samples were classified as per the AML96 study, and samples from high- and low-risk patients were excluded. High-risk patients had −5/del(5q), −7/del(7q), hypodiploid karyotypes (except 45, X, −Y, or −X), inv(3q), abnl12p, abnl11q, +11, +13, +21, +22, t(6;9); t(9;22); t(9;11); t(3;3), or multiple aberrations. Low-risk patients had t(8;21).

Cell lines

Human AML cell lines HL-60, Kasumi-1, KG-1a, ME-1, MOLM-13, MV4-11, NB-4, OCI/AML3, THP-1, U-937, ALL-derived Jurkat, chronic myeloid leukemia in blast crisis-derived K-562, and cervix carcinoma HeLa (ATCC, LGC Standards, Wesel, Germany) were cultured in RPMI 1640 containing 10% fetal calf serum (FCS), except for OCI/AML3 cells, which were cultured in minimum essential medium−α containing 20% FCS. Exon 12 *NPM1* gene mutational status was described for all cell lines, except for K562 [37]. In K562, the exon 12 *NPM1* wild-type was found as described previously [12]. All-*trans* retinoic acid (ATRA; Sigma Aldrich, Munich, Germany) and tumor necrosis factor−related apoptosis-inducing ligand (TRAIL; Alexion, Cheshire, CT, USA) were used in concentrations of 2 μM and 100 ng/mL.

miRNA extraction

Total RNA isolation and small RNA enrichment were performed using the miRVana miRNA isolation kit (Ambion, Austin, TX, USA) according to manufacturer's instructions.

miRNA labeling and microarray analyses

Labeling and microarray hybridization were performed as described previously [38]. Briefly, purified miRNAs were labeled using the miRVana miRNA labeling kit (Ambion) and amine-reactive dyes, as recommended by the manufacturer. Poly(A) polymerase and a mixture of unmodified and amine-modified nucleotides were used to append a polynucleotide tail to the 3′ end of each miRNA. The amine-modified miRNAs were coupled to NHS ester-modified Cy5 or Cy3 dyes (Amersham Bioscience, Piscataway, NJ, USA).

Unincorporated dyes were removed by a second glass-fiber filter-based cleaning procedure. Samples were independently labeled with Cy5 or Cy3 dyes, respectively. Absolute signal intensities for each spot were converted to relative intensities and converted to logarithm base 2 as Log2(Cy5/Cy3). Each array was then centered by correcting the mean Log2(Cy5/Cy3) ratio to zero to ensure all means of distributions of Log2(Cy5/Cy3) within arrays is zero. This process was performed for each array.

Samples were hybridized for 12 to 16 hours at 42°C on prespotted glass slides. Thymus miRNA served as a hybridization control. For analysis of miRNAs in clinical samples, a microarray platform detecting 203 miRNA species was used (for detailed information see Supplementary Table E1; online only, available at www.exphem.org). Following hybridization, the slides were scanned using a GenePix 4000B array scanner (MDC, Palo Alto, CA, USA), and each element was located and analyzed using the GenePix Pro 5.0 software package (Molecular Devices, Sunnyvale, CA, USA).

qRT-PCR

qRT-PCR was performed according to manufacturer's instructions (Ambion) using approximately 20 pg miRNA per assay. Primer pairs included those for *miR-10a* and the murine double minute 4 (*MDM4*) gene (all primers were from Ambion). For the *MDM4* gene, we used assay Hs00159092_m1, amplifying parts of the exon 3 and 4 of the major splice variant of the *MDM4* gene (NM_2393.3, ENST00000367182).

Because there is no generally accepted normalization control for miRNA quantification, we investigated the impact of four different miRNAs as potential normalization controls (*5S, miR-24, miR-93,* and *miR-103*). Best correlations with the data observed in microarray experiments were determined using datasets normalized to *5S* RNA (Supplementary Table E2; online only, available at www.exphem.org). We chose *5S* as the normalization control for miRNA for further quantification. mRNA expression was normalized to glyceraldehyde 3-phosphate dehydrogenase expression in the individual samples. The linearity of the assays was measured by dilution curve analysis, and the specificity of the assays was evaluated by melting curve analysis. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method [39].

Mutation detection

Mutations in the juxtamembrane domain of *FLT3* and exon 12 of *NPM1* were detected as described previously [9,12]. Patient samples were grouped according to the ratio of mutant (mut) to wild-type (wt) *FLT3*-ITD in three groups as described previously (no mutation; mut/wt *FLT3*-ITD $\leq$ 0.8; mut/wt *FLT3*-ITD $>$ 0.8) [9].

Transfection of leukemic cells

Cells were washed with phosphate-buffered saline and suspended in hypo-osmolar electroporation buffer (Eppendorf, Hamburg, Germany) at 1.5×10^7 cells/mL, then *pre–miR-10a*, pre-miR precursor molecule-negative control, *anti–miR-10a*, anti-miR precursor molecule-negative control, or siAURKB (all 50 nmol), or siGAPDH (100 pmol) (all Ambion) were added to 100 µL aliquots OCI/AML3 or K562 cells. Cells were electroporated with one pulse at 240 V for 100 µs. These cells remained in the electroporation cuvettes for 5 to 10 minutes prior to culture for 24 and 48 hours in RPMI 1640, containing 10% FCS or minimum essential medium−α containing 20% FCS. A small RNA-expressing GFP (a generous gift of Dr. Brenner, Department of Pediatrics, Dresden University of Technology, Dresden, Germany) was used to determine transfection efficiency in OCI/AML3 and K562 cells prior to studies using *miR-10a* antisense or overexpression strategies. Transfection efficiency was determined by propidium iodide−based fluorescence-activated cell sorting analysis with transfection efficiencies of 50% to 60% in K562 cells and 20% for OCI/AML3 cells.

MTT assay

The MTT (3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromid) proliferation assay was performed as described previously [40].

DNA oligonucleotide microarray

DNA oligonucleotide microarrays (Affymetrix, Carlsbad, CA, USA) were performed as described previously [40].

Cloning of the miR-10a binding site from 3′ UTR of MDM4

The putative *miR-10a* binding site within the 3′ UTR of *MDM4* was identified using the Targetscan algorithm (targetscan.org) based on the NCBI transcript NM_002393.2. The wt or mutated binding sites, respectively, were cloned into the pMIRReporter vector (Ambion) via the *Spe*I and *Hind*III sites using the oligonucleotides (Eurofins MWG Operon, Ebersberg, Germany) described in Table 1. Mutations of the binding site were performed by inverting the binding site (mutant 1) or by mutation of the seed sequence (mutant 2). The identity of all clones was verified by sequence analysis (Sequencing facility, MPI-CBG Dresden, Germany). The sequences of the used oligonucleotides are shown in Table 1.

Table 1. Sequences of used oligonucleotides

Wild-type sense	*Spe*I *Hind*III
	5′ - CTAGTGCTCAGCGGGAGGTGTGGGGCG**ACAGGGT**CA-3′
	3′ - ACGAGTCGCCCTCCACACCCCGC**TGTCCCA**GTTCGA-5′
Wild-type antisense	5′ - AGCTTGACCCTGTCGCCCCACACCTCCCGCTGAGCA -3′
Mutant 1 sense	*Spe*I *Hind*III
	5′ - CTAGTGCTCAGCCCCTCCACACCCCGC**TGTCCCA**GA-3′
	3′ - ACGAGTCGGGGAGGTGTGGGGCG**ACAGGGT**CTTCGA-5′
Mutant 1 antisense	5′ - AGCTTCTGGGACAGCGGGGTGTGGAGGGGCTGAGCA -3′
Mutant 2 sense	*Spe*I *Hind*III
	5′ - CTAGTGCTCAGCGGGAGGTGTGGGGCG**AAAAAAA**CA -3′
	3′ - ACGAGTCGCCCTCCACACCCCGC**TTTTTTT**GTTCGA-5′
Mutant 2 antisense	5′ - AGCTTGTTTTTTTCGCCCCACACCTCCCGCTGAGCA -3′

miR-10a seed match sequence in bold, mutated sequences are highlighted in grey background, and underlined type indicates the SpeI and HindIII restriction sites.

Transfection of MDM4 recombinants and luciferase assay

HeLa cells (2×10^5 cells/well) were seeded in 24-well plates (0.5 mL/well), and cotransfection of *MDM4* constructs (3′UTR/_wt/_mt1/_mt2) (0.4 μg/mL), pre−miR-control-, anti−miR-control-, pre−miR-10a-, anti−miR-10a-miRNA (50 nmol), and Renilla (pRL-TK plasmid; Promega, Mannheim, Germany) (80 ng/mL) was performed in triplicates using Lipofectamine 2000-based transfection (Invitrogen, Carlsbad, CA, USA). At 48 hours after transfection, cells were lysed with passive lysis buffer (Promega) and the supernatant was collected. The Dual-Luciferase Reporter Assay System (Promega) was used according to manufacturer's instructions. Luminescence was measured using the Mithras LB 940 multimode reader (Berthold Technologies, Bad Wildbad, Germany). Pre−miR-control values were normalized; pre- and anti−miR-10a values were calculated in relation to the normalized pre−miR-control values and expressed as a relative percentage value.

Western blots

Proteins were isolated as described previously [41], and electrophoresis was performed by loading 30 μg protein onto 12% acrylamide gels. Nitrocellulose membranes (BD Biosciences, San Jose, CA, USA) were incubated with primary antibodies specific for MDM4, glyceraldehyde 3-phosphate dehydrogenase, and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 1:500) overnight at 4°C. Blots were washed and incubated with horseradish peroxidase-goat anti-mouse or anti-rabbit secondary antibodies (Dako, Glostrup, Denmark; diluted 1:2000). Western blots were developed by chemiluminescence using the ECL Plus Chemiluminescence Detection Kit (GE Healthcare, Uppsala, Sweden).

Statistical analysis

miRNA data were filtered for quality and significance using the Longhorn Array Database [42]. Filters were based on minimum intensity and pixel consistency. All data used for analysis had a signal-to-noise ratio >5, an average sum intensity of 50% higher than that of the negative control spots, and a regression ratio >0.5. Data were normalized globally per array such that the average LogRatio was 0 after normalization.

Hierarchical clustering of expression of 203 miRNAs was performed with average linkage and Pearson correlation. For the determination of differentially expressed miRNAs in leukemic (L) and normal-like (NL) samples, a two-sample *t*-test assuming equal variance was performed for every miRNA, and multiplicity correction [43] was performed to control the false discovery rate at 0.05%. Analysis of variance tests for the determination of differentially expressed miRNAs were performed using the Web-based GEPAS system [44].

Clinical variables of statistical significance were compared using the χ^2-test for dichotomized variables and the Mann-Whitney *U*-test or Kruskal-Wallis H-test for continuous variables. Nonparametric correlations were performed to determine the correlation coefficient according to the Spearman rho test. Logistic regression analysis using multiple parameters was performed to identify the impact of single variables on the complete remission rate. Cox regression analysis was used to identify independent parameters associated with overall survival and relapse-free survival. The *p* values are two-sided, and a significance level of 0.05 was used. Clinical analyses were performed using SPSS version 16.0.1 (SPSS Inc, Chicago, IL, USA).

Results

Microarray analysis of miRNAs from AML (IR) patients

We compared relative expression of 203 miRNAs in 21 AML samples with (IR) cytogenetics (for patients characteristics see Supplementary Table E3; online only, available at www.exphem.org) with that of miRNAs in normal BM samples from five normal stem cell donors. Hierarchical cluster analysis demonstrated that most AML (IR) samples exhibited expression patterns different from those exhibited by normal BM samples (Fig. 1A). However, 5 of the 21 AML (IR) samples were not statistically different from normal BM samples. These samples were denoted as AML with NL miRNA expression and were subsequently compared with the L samples. Four of the five NL samples showed that the patients had *NPM1* mutation, despite the fact that they did not carry *FLT3*-ITD (Supplementary Table E3; online only, available at www.exphem.org). Fourteen miRNAs were then identified with differential expression levels between NL and L samples (Fig. 1B). Most of these miRNAs were downregulated in the L samples compared with the NL samples. A number of miRNAs resembled candidates with previously annotated functional impact, such as *miR-10a, miR-16, miR-221, miR-223* and members of the oncomiR cluster on chromosome 13, including *miR-17-5p* and *miR-20* (Table 2). Results were validated comparing AML samples with normal hematopoietic cells by qRT-PCR. Here, the extraordinary high expression of *miR-10a* in AML samples could be verified, which is even higher, as in normal $CD34^+$ cells. In contrast, expression differences for *miR-223* were more discrete and were therefore not studied further (see Supplementary Figure E1; online only, available at www.exphem.org).

miR-10a overexpression in AML (IR) patients with NPM1 mutations

We therefore validated the findings for *miR-10a* in a larger subset of 89 AML (IR) samples. We detected no correlation between *miR-10a* expression for various clinical and laboratory parameters, albeit *miR-10a* expression strongly correlated with the presence of *NPM1* mutations in all patients (Table 3), although the highest expression levels were observed in patients who lacked *FLT3*-ITD mutation, despite being positive for *NPM1* mutation (Fig. 2). As expected, high *miR-10a* expression had a strong negative correlation with expression of the CD34 antigen on blast samples. We conclude that there is an unusually strong *miR-10a* overexpression in *NPM1* mutant AML samples.

Functional significance of elevated miR-10a expression

We characterized a subset of leukemic and nonleukemic cells with respect to *miR-10a* and *MDM4* expression. Investigation of cell lines for *miR-10a* demonstrated a marked *miR-10a* overexpression in OCI/AML3 cells with mutated

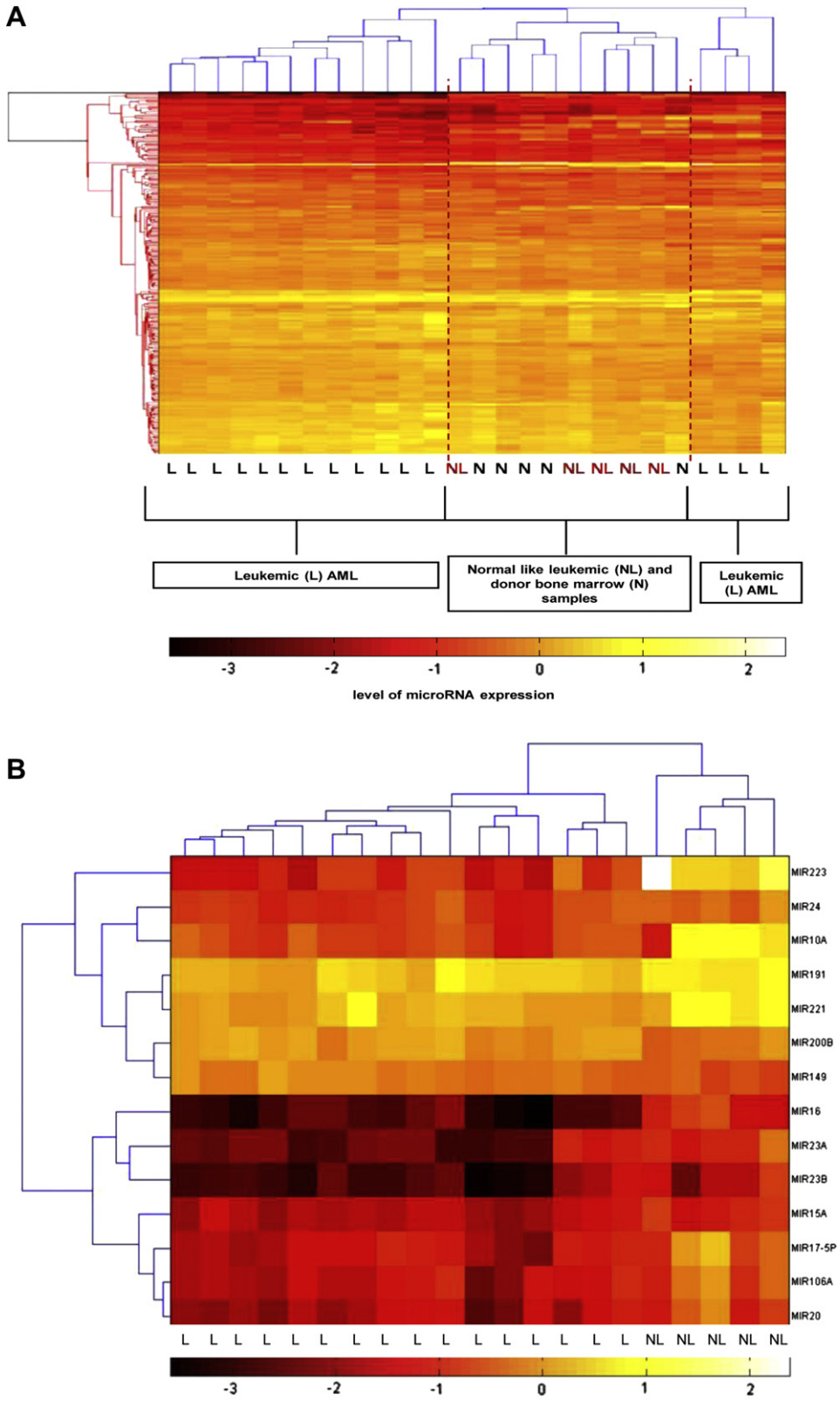

Figure 1. Differential miRNA expression in AML samples with (IR) cytogenetics. Hierarchical clustering of miRNA expression in AML samples with (IR) cytogenetics (L + NL) compared with that in normal bone marrow samples (N), samples in columns, miRNAs in rows. AML samples were denoted as either AML samples with aberrant miRNA profile (L = leukemic) or AML samples with a normal-like miRNA profile (NL = normal-like). (**A**) Heat map of AML (IR) miRNA expression profiles for 14 differentially regulated miRNAs comparing L and NL conditions, differential expression was determined for miRNA expression using *t*-test statistics and false discovery rate corrections (see Material and Methods).

Table 2. Comparison between differentially expressed miRNAs in AML (L) and (NL) samples

miRNA	Expression in AML (L) samples	Chromosomal location	Function	Reference
miR-223	Down	Xq12	Differentiation in granulopoiesis	[27]
miR-24	Down		Inhibition of erythropoiesis, targeting of DHFR	[60,61]
miR-24-1		9q22.32		
miR-24-2		19p13.13		
mir-10a	Down	17q21.32	Tumor cell metastasis for *miR-10b* in breast cancer	[49]
mir-191	Down	3p21.31	Negative prognostic impact on AML patients	[29]
mir-221	Down	Xp11.3	Downregulation of KIT and p27 (KIP1) in hematopoiesis and erythroleukemia	[62,63]
mir-200B	Up	1p36.33	Regulation of E-cadherin	[64]
mir-149	Up	2q37.3	Upregulation of KCNAB1 and LOX in clear cell renal cell carcinoma	[65]
mir-16	Down		Regulation of BCL-2 in leukemia	[66]
miR-16-1		13q14.2		
miR-16-2		3q25.33		
mir-23a	Down	19p13.12	Hypoxia induced	[67]
mir-23b	Down	9q22.32	Regulation of HES1-mediated retinoic acid differentiation	[68]
mir-15a	Down	13q14.2	Regulation of BCL-2 in leukemia	[66]
mir-17-5p	Down	13q31.3	Part of an "oncomir" cluster, c-Myc-dependent E2F1 regulation	[69,70]
mir-106a	Down	Xq26.2	Downregulation of RB1 in carcinogenesis	[71]
mir-20a	Down	13q31.3	Part of an "oncomir" cluster, c-Myc-dependent E2F1 regulation	[69,70]

BCL-2 = B-cell CLL/lymphoma 2; DHFR = dihydrofolate reductase; HES1 = hairy/enhancer of split 1; L = leukemic; LOX = lysyl oxidase; NL = normal-like; RB1 = retinoblastoma 1.

NPM1 compared with other leukemic and nonleukemic cells with wt *NPM1* (Fig. 3A). In contrast, *MDM4* expression was lower in mutated OCI/AML3 cells than in other wt cells (Fig. 3B). To prove the effect of *miR-10a* overexpression by functional studies, we chose K562 cells for further studies because these cells express low endogenous levels of *miR-10a* (see also Fig. 4A). We conducted a microarray experiment to compare whole genome expression of K562 cells transfected with *pre–miR-10a* with that of cells transfected with a negative control miRNA. We identified 130 transcripts with at least 1.5-fold downregulation after *pre–miR-10a* transfection. In contrast, only 30 genes were

Table 3. Characteristics of 89 AML (IR) patients investigated for *miR-10a* expression

	mir-10a			
	n = 89	≤Median	>Median	*p* Value
Age 60 y or younger, n (%)	65 (73)	37 (57)	28 (43)	
Sex (female) (%)	37 (42)	19 (51)	18 (49)	
WBC, median (range)	64 (2.8−380)	48 (2.8−350)	84 (4.6−380)	
BM blast, median (range)	78 (15−96)	77 (15−96)	78.5 (39−95)	
FAB, n (%)				
M0	2 (2)	2 (100)	0 (0)	
M1	29 (33)	15 (52)	14 (48)	
M2	22 (25)	12 (55)	10 (45)	
M4	10 (11)	4 (40)	6 (60)	
M4eo	3 (3)	3 (100)	0 (0)	
M5a	17 (19)	7 (41)	10 (59)	
M5b	4 (5)	2 (50)	2 (50)	
RAEB-T	2 (2)	2 (100)	0 (0)	
de novo, n (%)	84 (94)	44 (52)	40 (48)	
MDS, n (%)	5 (6)	3 (60)	2 (40)	
CD34%, median (range)	14 (0−96)	41 (0−95)	3.5 (0−96)	<0.0001
CD14%, median (range)	8 (0−74)	8 (0−66)	7 (0−74)	
NPM1 mutant, n (%)	36 (40)	7 (19)	29 (81)	<0.0001
FLT3-ITD, n (%)	37 (42)	19 (51)	18 (49)	
FLT3/D835, n (%)	11 (12)	5 (45)	6 (55)	
Extramedullary manifestation, n (%)	15 (18)	3 (7)	12 (27)	0.01

FAB = French-American-British association; MDS = myelodysplastic syndrome; NPM1 = nucleophosmin 1; RAEB-T = refractory anemia with excess blasts in transformation; WBC = white blood cells.

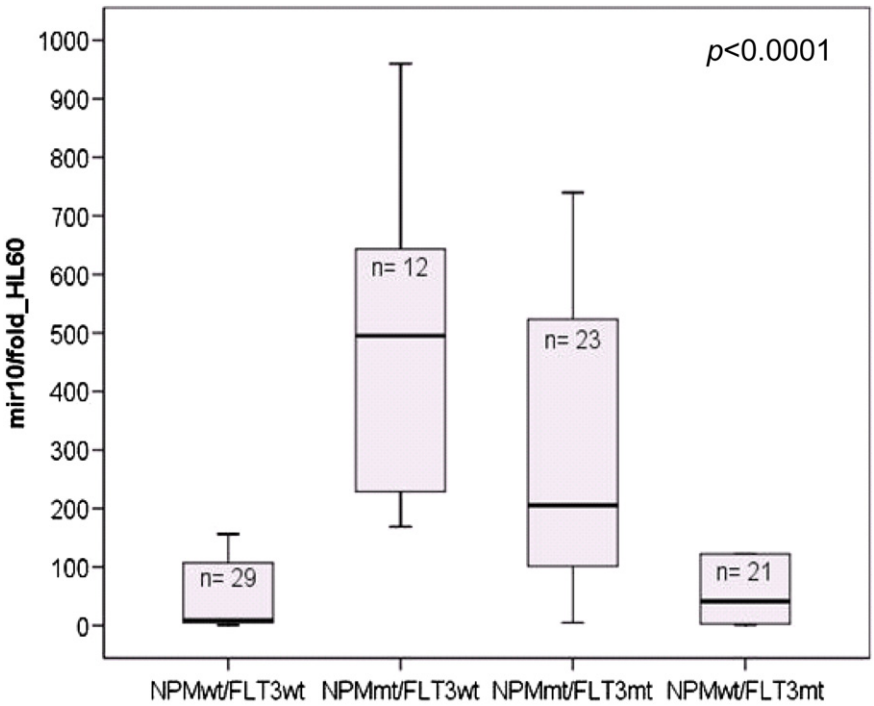

Figure 2. Expression of *mir-10a* in AML patients with IR cytogenetics according to their *NPM1* or *FLT3*-ITD mutational status. Expression levels of *mir-10a* in AML patients (n = 89) with IR cytogenetics; expression levels were determined by qRT-PCR using 5S as an internal control; values were calculated according to the $2^{-\Delta\Delta CT}$ method; the HL60 cell line served as an internal control; *NPMwt*, no mutation in exon12 of *NPM1*; *FLT3wt*, no TK or ITD mutation; *NPMmt*, mutated *NPM1*; *FLT3mt*, either TK or ITD mutation in *FLT3* ($p < 0.001$; Kruskal-Wallis H-test for continuous variables).

upregulated to >1.5-fold in cells transfected with *pre–miR-10a* (see Supplementary Table E4; online only, available at www.exphem.org).

One of the most strongly influenced genes downregulated by *pre–miR-10a* was *MDM4* (see Supplementary Table E4; online only, available at www.exphem.org). For validation, we next analyzed the expression levels of *MDM4* in HeLa and K562 cells after transient transfection of *pre–miR-10a*. Here, we observed that MDM4 protein levels were downregulated after *pre–miR-10a* transfection (Fig. 3C). To verify a direct effect of *miR-10a* on *MDM4* regulation, we cloned wt and two mutants of the putative *miR-10a* binding site out of the 3′UTR of *MDM4* into the 3′UTR of a luciferase gene and performed luciferase assays with *pre–* and *anti–miR-10a*-miRNA. Reporter vector containing the wt binding site out of the *MDM4* gene showed a reduction in luciferase activity after cotransfection with *pre–miR-10a*. This was not observed when reporter vectors with a mutated binding site were used, indicating a direct action of *miR-10a* on *MDM4* 3′UTR (Fig. 3D).

To further characterize functional consequences of differential *miR-10a* expression, we transfected OCI/AML3 cells with inhibitory *anti–miR-10a* molecules. *MiR-10a* modulation resulted in altered growth characteristics as compared to the respective control, as inhibition of *miR-10a* resulted in partial resistance to both TRAIL and ATRA treatment (Supplementary Figure E2; online only, available at www.exphem.org).

Finally, we investigated *MDM4* expression levels in AML (IR) patients (n = 143) with mutated *NPM1* (n = 70) as compared to those with wt *NPM1* gene (n = 73). *MDM4* expression levels in patients with a mutated *NPM1* gene showed a tendency for lower expression in comparison to wt *NPM1* (Fig. 4A; $p = 0.07$). Furthermore, Western blot analyses of 16 AML samples (mutated *NPM1*, n = 8 and *NPM1* wt, n = 8) demonstrated a clear reduction of MDM4 expression in most *NPM1* mutated samples (Fig. 4B).

Discussion

The detection of altered expression patterns of miRNAs in cancer patient samples may lead to development of important prognostic indicators and could potentially be used to direct treatment strategies on a case-by-case basis. Importantly, it was recently shown that miRNA expression can be used to distinguish AML and normal BM samples [45] from ALL and AML samples [31]. Results of our studies indicate that each leukemia sample can be classified according to particular mutation, which in turn alter expression of specific miRNAs. To exclude miRNA expression differences associated with chromosomal aberrations in AML patients, we investigated miRNA expression patterns in samples from AML patients with (IR) cytogenetics.

We used a microarray-based approach to screen for potential differences in miRNA expression in AML (IR) samples vs. normal BM samples. Heterogeneity of miRNA

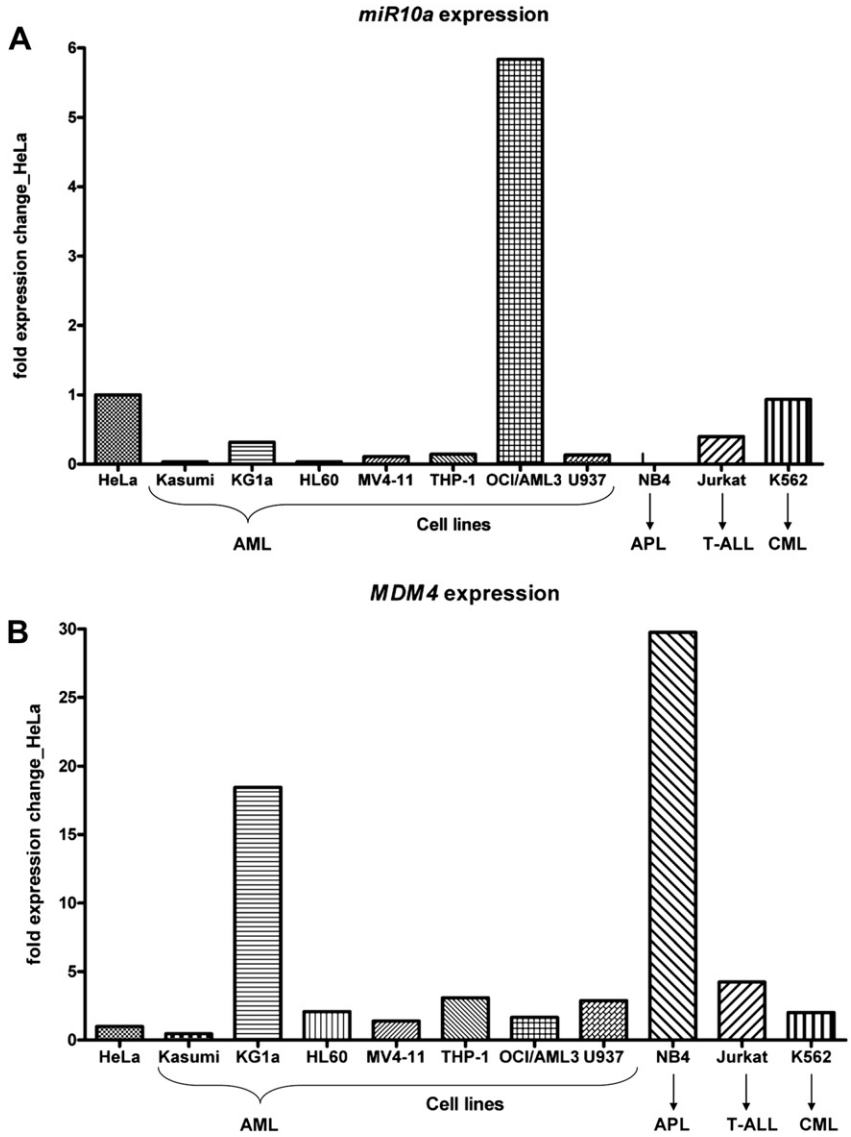

Figure 3. Functional importance of *miR-10a*. (**A**) Expression of *miR-10a* in different cell-lines was determined by qRT-PCR. Results are shown as relative units compared with the determination in HeLa cells. (**B**) Expression of *MDM4* in different cell lines was determined by qRT-PCR. Results are shown as relative units compared with the determination in HeLa cells.

expression in AML samples was evident. Although most samples showed aberrant miRNA expression, some AML (NL) samples were not easily distinguished from normal BM samples by microarray analysis. Similar results of hierarchical cluster analyses were reported by Mi et al., who showed that normal BM samples could form subclusters within the clusters of AML samples [31]. The authors concluded that their discriminatory miRNAs were rather deregulated in ALL samples in relative to normal control samples. Interestingly, the NL samples identified were associated with the presence of *NPM1* mutation. Proposed data and models exist favoring the hypothesis that *NPM1* mutations display "founder genetic alterations" defining a distinct AML entity, which in the absence of *FLT3*-ITD mutation is associated with a favorable prognosis [46]. This could indicate that AML samples, which cluster with normal BM samples, indicate "early" AML with only few alterations. Alternatively, this could be an argument for a higher differentiation state of the AML *NPM1* mutated samples.

However, the molecular consequences of mutated *NPM1* in AML are incompletely understood. Gene expression profiles of *NPM1* mutant samples have been compared with AML samples containing wt *NPM1* and a strong clustering effect of *NPM1* mutation was observed [18]. These data indicate that the transcriptional program of *NPM1* mutant samples is clearly different from other AML samples with IR characteristics. Distinctive miRNA signatures have been described in *NPM1* mutant AML samples with normal karyotype [47].

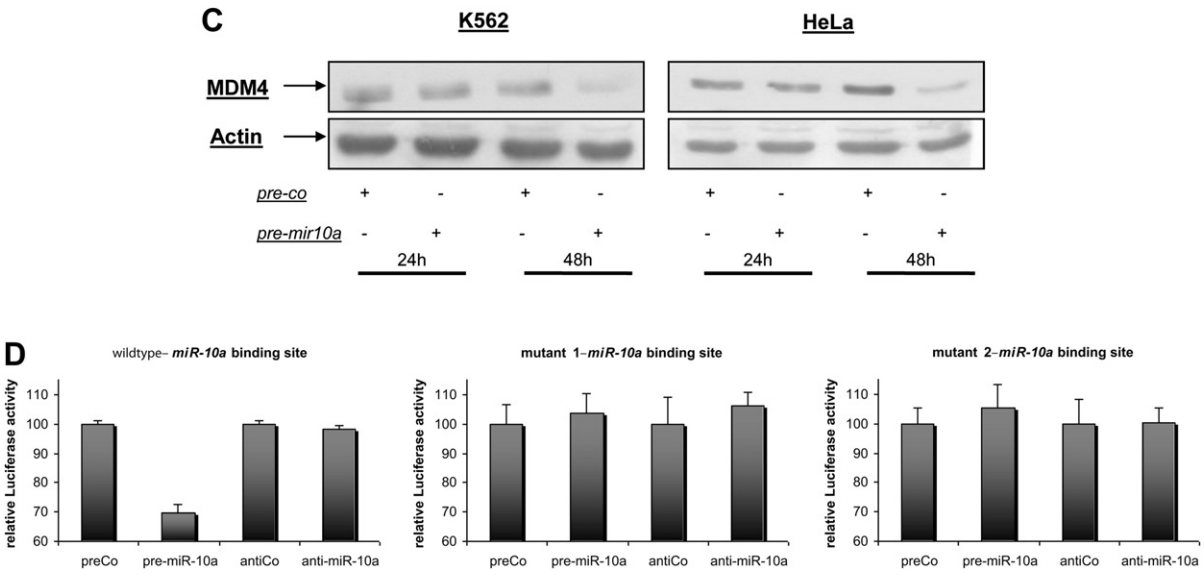

Figure 3. (*Continued.*) (**C**) Western Blot of HeLa and K562 cells transfected with either control pre-miRNA or *pre–miR-10a*. Cells were transfected with 50 nmol miRNA constructs using Amaxa technology (T19). After 24 and 48 hours protein was isolated. (**D**) Luciferase assays in HeLa cells using reporter vectors containing the wt binding site out of the *MDM4* 3′UTR or a mutated variant (mt1, mt2). The cells were cotransfected with the reporter vector and *pre–miR-control- (preCo)*, *anti–miR-control- (antiCo)*, *pre–miR-10a-*, and *anti–miR-10a-miRNA*. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Results for luciferase experiments shown, are representative for n = 5 independent experiments.

The results presented confirm previous findings by showing that *miR-10a* is specifically overexpressed in *NPM1* mutant AML samples [47]. Recently, Garzon et al. detected high *miR-10a* expression levels in $CD34^+$ hematopoietic progenitor cell samples, which decreased during the in vitro differentiation of megakaryocytes [48]. Moreover, a cluster of miRNAs, including *miR-10a*, was upregulated in AML patients with normal karyotype [34].

Ma et al. found that *miR-10b* is involved in migration of malignant cells, resulting in augmented metastasis rates [49]. *NPM1* mutations are known to correlate with extramedullary involvement (e.g., leukemic manifestations in soft tissue, skin, or organs) in AML [10]. Analyzing our 89 AML (IR) patient samples in which *miR-10a* expression was investigated, we found 15 patients (18%) with extramedullary manifestation of AML at diagnosis, of which 13 patients (87%) harbored mutated *NPM1* (Table 3).

MiR-10a overexpression in *NPM1* mutant AML samples could be a consequence of the transcriptional regulation of a stem cell program. Several groups have described gene expression patterns in *NPM1* mutant AML samples [18,19] and found that *NPM1* mutations are associated with the transcriptional activation of *homeobox* (*HOX*) gene clusters. *MiR-10a* is located within *HOXB* cluster (between *HOXB4* and *HOXB5*) and, as this location is conserved even in invertebrates, it indicates functionality of the genetic region [50]. Mansfield et al. [50] observed coexpression of *HOXB4* and *miR-10a* (located 3-prime to *HOXB4*) in developing mouse embryos, and concluded that *miR-10a* and *HOXB* genes were possibly regulated by common transcriptional mechanisms. Consistent with previous studies, we observed a strong association between *miR-10a* and *HOXB4* expression in *NPM1* mutant samples (data not shown) [47].

There have only been a limited number of reports that define a specific biological property for *miR-10a*. To identify potential target genes, we conducted microarray studies that detected *MDM4* downregulation. Thus, one explanation for the observed effect, i.e., *miR-10a* overexpression in *NPM1* mutant AML samples can impact cellular defense mechanisms, is *MDM4* downregulation, which is a known mediator of cellular stress that interferes with p53 activation. Because normal NPM1 function includes stabilization of p53 in the nucleus upon DNA damage [51], it has been suggested that mutated *NPM1* might interfere with these properties [6]. TRAIL induces apoptosis in malignant cells, but not normal cells, while ATRA inhibits cell proliferation and induces differentiation and apoptosis in various ways [52,53]. We could show that partial resistance to both TRAIL and ATRA treatment was induced by *miR-10a* modulation in *NPM1*-mutated OCI/AML3 cells.

MDM4 and MDM2 are critical cellular proteins that balance p53 activation in a nonredundant manner (for review see [54]). MDM4 has been shown to repress p53-mediated transcriptional activation apparently by a direct interaction with p53 at its promoter binding sites [55]. This activity is in contrast to MDM2-mediated p53 degradation and may explain the nonredundant effects of interfering with the two structurally related proteins. There is currently no information on MDM4 expression and activity in AML patients. In contrast, the effect of mutated NPM1 may depend in part of its inhibitory effect on the

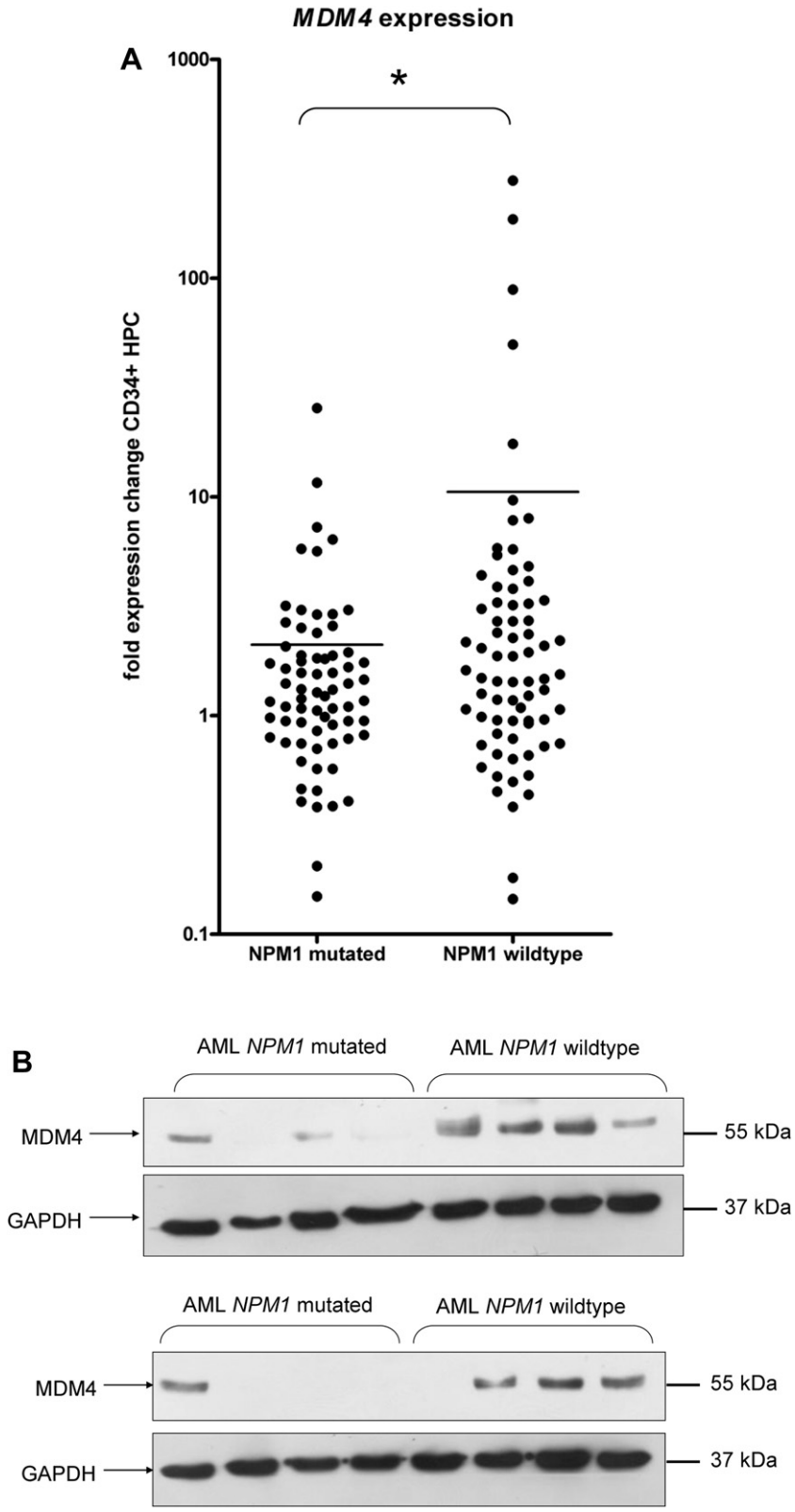

Figure 4. Distribution of *miR-10a* and *MDM4* in AML. (**A**) Expression of *MDM4* mRNA in AML patients with *NPM1* mutations (NPM1 mt., n = 70) and wt *NPM1* (NPM1 wt., n = 73), determined by qRT-PCR. Results are shown as relative units compared to the expression in $CD34^+$ HPCs. *$p = 0.07$; unpaired two-tailed *t*-test. (**B**) Expression of MDM4 protein in AML patients with *NPM1* mutations (NPM1 mt., n = 8) and wt *NPM1* (NPM1 wt., n = 8), determined by Western blot analysis.

tumor suppressor ARF, which has been shown to localize in the cytoplasm in association with mutated NPM1. However, additional effects of mutated NPM1 were suspected in this study [56]. Therefore, MDM4 inactivation could be one explanation for the observed complex activity of mutated *NPM1*. Recently, Harutyunyan et al. demonstrated that chromosome 1q amplifications, harboring the *MDM4* gene, in postmyeloproliferative AML were significantly associated with transformation to AML as compared to chronic-phase myeloproliferative neoplasms [57]. Furthermore, elevated protein levels of the two p53-regulators, MDM2 and MDM4 have been shown to influence the sensitivities of MDM2 inhibitors, such as Nutlins and the MI-series [58]. Because there exist multiple molecular mechanisms that influence the sensitivity and resistance to MDM2 inhibitors, the regulatory mechanisms through miRNAs in this context warrant further investigation [59]. Because our analysis of AML (IR) patients showed a tendency toward lower *MDM4* gene expression data in patients with mutated *NPM1* but did not reach statistical significance, we speculate that there may exist other influencing—yet to be discovered—factors that might contribute to these pathophysiological mechanisms.

In conclusion, our results indicate that AML patients with IR characteristics and miRNA array clusters similar to that of normal BM donors, harboring *NPM1* mutations have high *miR-10a* expression levels. Finally, we identified *MDM4* to be a target of *miR-10a* in patients with *NPM1* mutations, which has been delineated in AML for the first time, to the best of our knowledge. Based on these studies the clinical impact of *miR-10a* and *MDM4* in AML warrants further investigations.

Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

Acknowledgments

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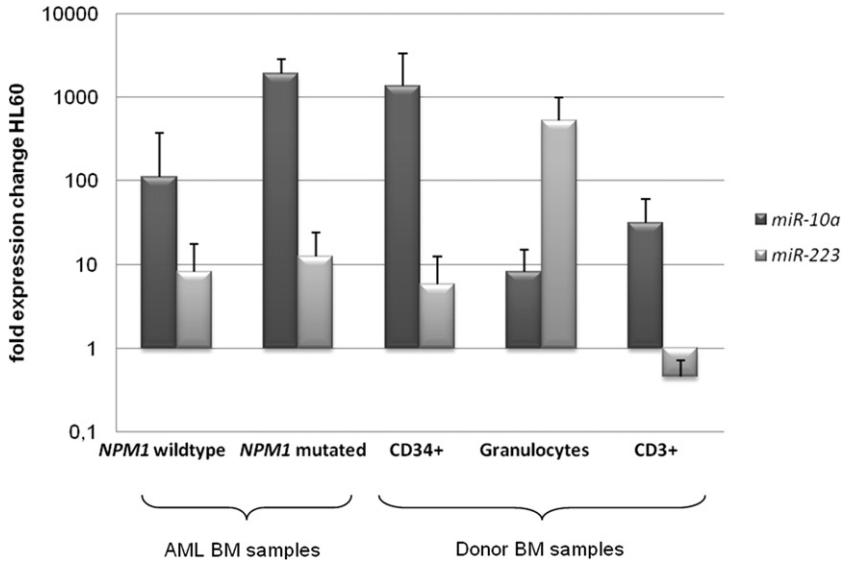
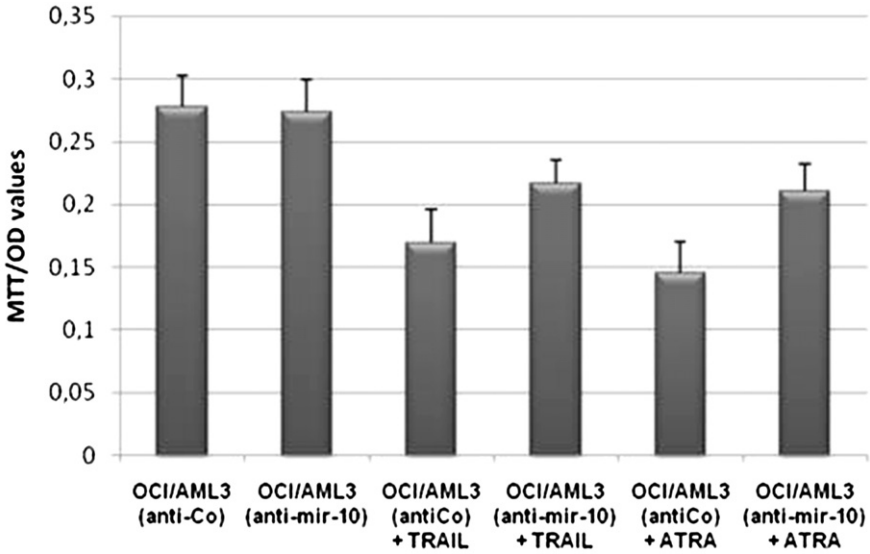


Supplementary Figure E1. *MiR-10a* and *miR-223* expression with qRT-PCR AML in AML bone marrow (BM) samples (*NPM1* mutated, n = 7; AML *NPM1* wild-type, n = 8) and healthy BM donors ($CD34^+$ [isolated], n = 9; granulocytes, n = 10; and $CD3^+$ T cells, n = 10).



Supplementary Figure E2. Influence of external stimuli on OCI/AML3 cells. Cells were transfected with *anti–miR-control* (anti-Co) or *anti–miR-10a*, after 24 hours cells were exposed to either TRAIL or ATRA. After an additional 24 hours, MTT determination was performed.

Supplementary Table E1. Investigated miRNAs as spotted on arrays for AML samples with IR cytogenetics

LET7A
LET7B
LET7C
LET7D
LET7D-AS
LET7E
LET7F-1
LET7F-2
LET7G
LET7I
MIR1
MIR100
MIR101
MIR103
MIR105
MIR106A
MIR106B
MIR107
MIR10A
MIR122A
MIR124A
MIR125A
MIR125B-1
MIR126
MIR126-AS
MIR127
MIR128A
MIR129
MIR130A
MIR130B
MIR132
MIR133A
MIR134
MIR135A
MIR136
MIR137
MIR138
MIR139
MIR140
MIR141
MIR142-3P
MIR142-5P
MIR143
MIR144
MIR145
MIR146
MIR147
MIR148A
MIR149
MIR150
MIR151
MIR152
MIR153
MIR154
MIR155
MIR15A
MIR15B
MIR16
MIR17-3P
MIR17-5P
MIR18
MIR181A

(*continued*)

Supplementary Table E1. (*continued*)

MIR181B
MIR182
MIR182-AS
MIR183
MIR184
MIR185
MIR186
MIR187
MIR188
MIR189
MIR190
MIR191
MIR192
MIR193
MIR194
MIR195
MIR196-2
MIR198
MIR199A
MIR199A-2
MIR199A-2-AS
MIR199A-AS
MIR19A
MIR20
MIR200A
MIR200B
MIR201
MIR202
MIR203
MIR204
MIR205
MIR206
MIR207
MIR208
MIR21
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MIR215
MIR216
MIR217
MIR218
MIR219
MIR22
MIR221
MIR222
MIR223
MIR224
MIR23A
MIR23B
MIR24
MIR25
MIR26A
MIR27A
MIR28
MIR290
MIR291-3P
MIR291-5P
MIR292-3P
MIR292-5P
MIR293

(*continued*)

Supplementary Table E1. (*continued*)

MIR294
MIR295
MIR296
MIR297-1
MIR298
MIR299
MIR29B
MIR300
MIR301
MIR302
MIR302B-AS
MIR302C
MIR302C-AS
MIR30A
MIR30A-AS
MIR30B
MIR31
MIR32
MIR320
MIR322
MIR323
MIR324-3P
MIR324-5P
MIR325
MIR326
MIR328
MIR33
MIR330
MIR331
MIR335
MIR337
MIR338
MIR339
MIR340
MIR341
MIR342
MIR344
MIR345
MIR346
MIR34A
MIR34C
MIR350
MIR351
MIR361
MIR367
MIR368
MIR369
MIR370
MIR371
MIR372
MIR373
MIR373-AS
MIR374
MIR376A
MIR376B
MIR380
MIR381
MIR382
MIR383
MIR384
MIR409
MIR410
MIR411

(*continued*)

Supplementary Table E1. (*continued*)

MIR412
MIR422A
MIR423
MIR424
MIR425
MIR7
MIR9
MIR92
MIR93
MIR95
MIR96
MIR98
MIR99A
MIR99B
MIR9-AS

Supplementary Table E2. Comparison of qRT-PCR data with semi-quantitative data obtained from microarray datasets

Correlation	Array data	miR-150	miR-16	miR-223	miR-23a
miRVana 5S Norm		0.091595	0.502172	0.543302	0.547198
PEP mir-24 Norm		0.174745	0.307471	0.21967	−0.104351
PEP mir-103 Norm		0.057825	0.395851	0.160868	−0.120495
PEP mir-93 Norm		−0.042018	0.410549	0.19493	−0.033862

Data are shown as correlation coefficients.

Supplementary Table E3. Characteristics of AML (IR) patients

Patient ID	Array group	Age (y)	Sex	FAB subtype	BM blasts (%)	WBC count at diagnosis (Gpt/L)	Karyotype	FLT3-ITD	FLT3-ITD mt/wt ratio	NPM1-mt
29	Leukemic	54	M	M4	92.00	224.00	46XY	Neg	NA	Pos
30	Leukemic	34	M	M1	91.50	104.00	46XY	Neg	NA	Neg
31	Leukemic	22	F	M1	91.50	22.60	46,XX	Neg	NA	Neg
32	Leukemic	28	M	M1	90.50	58.60	45,X,-Y	Neg	NA	Neg
33	Leukemic	36	M	M1	89.50	260.00	46,XY	Neg	NA	Neg
34	Leukemic	33	M	M5a	89.50	120.00	46,XY,del(10)(p1?3)	Neg	NA	Neg
35	Leukemic	46	M	M5a	88.00	41.37	46,XY,+Y,+mar	Neg	NA	Neg
37	Leukemic	59	M	M5a	83.00	182.60	46,XY	Neg	NA	Pos
39	Leukemic	55	M	M4	80.00	3.90	47,XY	Pos	.04	Neg
40	Leukemic	33	F	M5a	88.00	131.00	46,XX	Pos	4.6	Neg
41	Leukemic	49	F	M2	90.00	153.00	46,XX	Pos	.85	ND
42	Leukemic	47	F	M1	93.50	72.30	46,XX	Pos	23.4	Pos
43	Leukemic	41	M	M1	86.00	78.30	46,XY	Pos	.72	Neg
45	Leukemic	52	M	M1	86.00	50.00	46,XY	Pos	.13	Pos
46	Leukemic	24	M	M1	96.00	148.00	Unknown*	Pos	.81	Neg
47	Leukemic	22	M	M1	90.00	29.63	47,XY,+4	Pos	.86	Neg
27	Normal-like	51	M	M5a	95.00	120.00	46,XY	Neg	NA	Pos
28	Normal-like	39	F	M1	93.00	380.00	46,XY	Neg	NA	Pos
36	Normal-like	60	M	M1	86.50	54.95	46,XY	Neg	NA	Pos
38	Normal-like	57	M	M1	81.50	130.00	Unknown*	Neg	NA	Pos
44	Normal-like	18	F	M5a	91.50	86.70	47,XX,+8	Pos	1.67	Neg

F = female; FAB = French-American-British association; FLT3 = fms-like tyrosine kinase 3; ITD = internal tandem duplication; mt/wt-ratio = mutant-to-wild-type-ratio; M = male;
NPM1 = nucleophosmin 1; WBC = white blood cells.
*Patients were defined as AML (IR) by performing fluorescence in situ hybridization analysis excluding high-risk cytogenetic features

Supplementary Table E4. Deregulated mRNAs

Accession ID	Fold change ([pre-mir-10a] vs. [pre-co])	Regulation ([pre-mir-10a] vs. [pre-co])	Gene symbol	Gene title
211022_s_at	1,4995008	Down	*ATRX*	Alpha thalassemia/mental retardation syndrome X-linked (RAD54 homolog)
221078_s_at	1,499976	Down	*CCDC88A*	Coiled-coil domain containing 88A
200722_s_at	1,5007571	Down	*CAPRIN1*	Cell cycle−associated protein 1
217202_s_at	1,5011952	Down	*GLUL*	Glutamate-ammonia ligase (glutamine synthetase)
213286_at	1,5034212	Down	ZFR	Zinc finger RNA binding protein
216855_s_at	1,5044638	Down	HNRNPU	Heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)
202479_s_at	1,5052016	Down	TRIB2	Tribbles homolog 2 (Drosophila)
204770_at	1,505952	Down	TAP2	Transporter 2
220386_s_at	1,506167	Down	EML4	Echinoderm microtubule associated protein like 4
219858_s_at	1,5078781	Down	FLJ20160	FLJ20160 protein
222283_at	1,5086538	Down	ZNF480	Zinc finger protein 480
210585_s_at	1,5097202	Down	TNPO2	Transportin 2 (importin 3)
220800_s_at	1,5097871	Down	TMOD3	Tropomodulin 3 (ubiquitous)
216549_s_at	1,5099028	Down	*TBC1D22B*	TBC1 domain family
208721_s_at	1,5122412	Down	ANAPC5	Anaphase promoting complex subunit 5
214390_s_at	1,5151955	Down	BCAT1	Branched chain aminotransferase 1
211273_s_at	1,516987	Down	TBX1	T-box 1
212008_at	1,5182364	Down	UBXD2	UBX domain−containing 2
211074_at	1,5187875	Down	FOLR1	Folate receptor 1 (adult)
214971_s_at	1,5205553	Down	ST6GAL1	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1
201835_s_at	1,5208024	Down	PRKAB1	Protein kinase
219558_at	1,5210507	Down	ATP13A3	ATPase type 13A3
213756_s_at	1,5210936	Down	HSF1	Heat shock transcription factor 1
213472_at	1,5231981	Down	HNRNPH1	Heterogeneous nuclear ribonucleoprotein H1 (H)
208650_s_at	1,5232625	Down	*CD24*	CD24 molecule
214849_at	1,5236279	Down	KCTD20	Potassium channel tetramerization domain containing 20
205966_at	1,524531	Down	TAF13	TAF13 RNA polymerase II
211094_s_at	1,5279856	Down	*NF1*	Neurofibromin 1
214245_at	1,5282876	Down	RPS14	Ribosomal protein S14
216521_s_at	1,5292673	Down	BRCC3	BRCA1/BRCA2-containing complex
215509_s_at	1,5362564	Down	BUB1	BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)
201902_s_at	1,5372647	Down	YY1	YY1 transcription factor
214190_x_at	1,5377111	Down	*GGA2*	Golgi associated
211220_s_at	1,538794	Down	HSF2	Heat shock transcription factor 2
207686_s_at	1,5416571	Down	*CASP8*	Caspase 8
215099_s_at	1,5421553	Down	RXRB	Retinoid X receptor
210932_s_at	1,5472378	Down	RNF6	Ring finger protein (C3H2C3 type) 6
201971_s_at	1,5487323	Down	ATP6V1A	ATPase
201151_s_at	1,5501565	Down	MBNL1	Muscleblind-like (Drosophila)
201337_s_at	1,550751	Down	VAMP3	Vesicle-associated membrane protein 3 (cellubrevin)
205835_s_at	1,5521927	Down	YTHDC2	YTH domain containing 2
211574_s_at	1,5548548	Down	CD46	CD46 molecule
214697_s_at	1,5671521	Down	ROD1	ROD1 regulator of differentiation 1 (Schizosaccharomyces *Pombe*)
205371_s_at	1,5726333	Down	*DBT*	Dihydrolipoamide branched chain transacylase E2
211090_s_at	1,5748426	Down	PRPF4B	PRP4 pre-mRNA processing factor 4 homolog B (yeast)
200841_s_at	1,5784054	Down	EPRS	Glutamyl-prolyl-TMA synthetase
201299_s_at	1,5804567	Down	MOBKL1B	Mob1
215739_s_at	1,5825475	Down	TUBGCP3	Tubulin
209456_s_at	1,5866083	Down	FBXW11	F-box and WD repeat domain containing 11
221268_s_at	1,5916929	Down	SGPP1	Sphingosine-1-phosphate phosphatase 1
211088_s_at	1,5917389	Down	PLK4	Polo-like kinase 4 (Drosophila)
205018_s_at	1,5934554	Down	MBNL2	Muscleblind-like 2 (Drosophila)
210077_s_at	1,5964437	Down	SFRS5	Splicing factor
210457_x_at	1,597052	Down	HMGA1	High mobility group AT-hook 1
214216_s_at	1,5996033	Down	LARP5	La ribonucleoprotein domain family
221638_s_at	1,6044447	Down	STX16	Syntaxin 16
207793_s_at	1,6099403	Down	EPB41	Erythrocyte membrane protein band 4.1 (elliptocytosis 1, RH-linked)
200952_s_at	1,6109558	Down	CCND2	Cyclin D2
219872_at	1,612254	Down	C4orf18	Chromosome 4 open reading frame 18

(*continued*)

Supplementary Table E4. (*continued*)

Accession ID	Fold change ([pre-mir-10a] vs. [pre-co])	Regulation ([pre-mir-10a] vs. [pre-co])	Gene symbol	Gene title
217445_s_at	1,6147336	Down	*GART*	Phosphoribosylglycinamide formyltransferase
207824_s_at	1,6155097	Down	MAZ	MYC-associated zinc finger protein (purine-binding transcription factor)
212574_x_at	1,6177799	Down	C19orf6	Chromosome 19 open reading frame 6
34478_at	1,6191995	Down	RAB11B	Rab11b
210317_s_at	1,6203048	Down	YWHAE	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein
216205_s_at	1,6223296	Down	MFN2	Mitofusin 2
203032_s_at	1,629594	Down	FH	Fumarate hydratase
206788_s_at	1,6300653	Down	*CBFB*	Core-binding factor
221628_s_at	1,6326449	Down	N-PAC	Cytokine-like nuclear factor n-pac
206241_at	1,6330737	Down	KPNA5	Karyopherin alpha 5 (importin alpha 6)
211205_x_at	1,6347475	Down	PIP5K1A	Phosphatidylinositol-4-phosphate 5-kinase
210866_s_at	1,6352849	Down	*CNOT4*	CCR4-NOT transcription complex
201559_s_at	1,6427157	Down	CLIC4	Chloride intracellular channel 4
204666_s_at	1,6431797	Down	*RP5-1000E10.4*	Suppressor of IKK epsilon
215236_s_at	1,6513644	Down	PICALM	Phosphatidylinositol binding clathrin assembly protein
208097_s_at	1,6527044	Down	TXNDC1	Thioredoxin domain containing 1
212619_at	1,6556174	Down	TMEM194	Transmembrane protein 194
216901_s_at	1,6561874	Down	IKZF1	IKAROS family zinc finger 1 (Ikaros)
201211_s_at	1,6571327	Down	DDX3X	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3
209006_s_at	1,6659354	Down	C1orf63	Chromosome 1 open reading frame 63
216985_s_at	1,668429	Down	STX3	Syntaxin 3
204427_s_at	1,6690917	Down	*TMED2*	Transmembrane emp24 domain trafficking protein 2
214007_s_at	1,6702452	Down	TWF1	Twinfilin
220797_at	1,6732249	Down	METT10D	Methyltransferase 10 domain containing
215220_s_at	1,6778793	Down	TPR	Translocated promoter region (to activated MET oncogene)
218748_s_at	1,6789892	Down	*EXOC5*	Exocyst complex component 5
202199_s_at	1,687683	Down	SRPK1	SFRS protein kinase 1
210828_s_at	1,6894844	Down	**ARNT**	**Aryl hydrocarbon receptor nuclear translocator**
200917_s_at	1,6923304	Down	SRPR	Signal recognition particle receptor ('docking protein')
206665_s_at	1,706015	Down	*BCL2L1*	BCL2-like 1
212105_s_at	1,7068222	Down	DHX9	DEAH (Asp-Glu-Ala-His) box polypeptide 9
216915_s_at	1,7102363	Down	PTPN12	Protein tyrosine phosphatase
211547_s_at	1,7120155	Down	*PAFAH1B1*	Platelet-activating factor acetylhydrolase
212392_s_at	1,7138575	Down	LOC652526///PDE4DIP	Phosphodiesterase 4D interacting protein (myomegalin)///similar to phosphodiesterase 4D interacting protein isoform 2
214130_s_at	1,7150333	Down	PDE4DIP	Phosphodiesterase 4D interacting protein (myomegalin)
211162_x_at	1,7171289	Down	SCD	Stearoyl-coa desaturase (delta-9-desaturase)
211016_x_at	1,7218277	Down	HSPA4	Heat shock 70-kda protein 4
217859_s_at	1,7238463	Down	SLC39A9	Solute carrier family 39 (zinc transporter)
217870_s_at	1,7274396	Down	*CMPK1*	Cytidine monophosphate (UMP-CMP) kinase 1
205867_at	1,7283465	Down	*PTPN11*	Protein tyrosine phosphatase, nonreceptor type 11
206184_at	1,7298421	Down	CRKL	V-crk sarcoma virus CT10 oncogene homolog (avian)-like
213548_s_at	1,7347237	Down	CDV3	CDV3 homolog (mouse)
214071_at	1,7373677	Down	MPPE1	Metallophosphoesterase 1
208116_s_at	1,7457724	Down	MAN1A1	Mannosidase
204560_at	1,7470237	Down	*FKBP5*	FK506 binding protein 5
216493_s_at	1,7531601	Down	IGF2BP3///LOC645468	Insulin-like growth factor 2 mRNA binding protein 3///similar to putative RNA binding protein KOC
215581_s_at	1,754282	Down	MCM3AP	Minichromosome maintenance complex component 3−associated protein
208047_s_at	1,7557199	Down	NAB1	NGFI-A binding protein 1 (EGR1 binding protein 1)
200796_s_at	1,7608273	Down	MCL1	Myeloid cell leukemia sequence 1 (BCL-2−related)
210440_s_at	1,7636423	Down	*CDC14A*	CDC14 cell division cycle 14 homolog A (Saccharomyces *Cerevisiae*)
203626_s_at	1,7687414	Down	SKP2	S-phase kinase-associated protein 2 (p45)
210892_s_at	1,7765058	Down	GTF2I	General transcription factor II
209629_s_at	1,7871082	Down	*NXT2*	Nuclear transport factor 2-like export factor 2
209754_s_at	1,7933791	Down	TMPO	Thymopoietin
216902_s_at	1,8083738	Down	LOC653390/// LOC730092///RRN3	RRN3 RNA polymerase I transcription factor homolog (*Saccharomyces Cerevisiae*)///RRN3 RNA polymerase I transcription factor homolog (*Saccharomyces Cerevisiae*) pseudogene

(*continued*)

Supplementary Table E4. (*continued*)

Accession ID	Fold change ([pre-mir-10a] vs. [pre-co])	Regulation ([pre-mir-10a] vs. [pre-co])	Gene symbol	Gene title
217176_s_at	1,809362	Down	ZFX	Zinc finger protein
205732_s_at	1,8224169	Down	NCOA2	Nuclear receptor coactivator 2
215150_at	1,8244326	Down	*YOD1*	YOD1 OTU deubiquinating enzyme 1 homolog (*S. Cerevisiae*)
212142_at	1,8362669	Down	MCM4	Minichromosome maintenance complex component 4
214786_at	1,8539861	Down	MAP3K1	Mitogen-activated protein kinase kinase kinase 1
219608_s_at	1,854201	Down	FBXO38	F-box protein 38
217097_s_at	1,8662989	Down	PHTF2	Putative homeodomain transcription factor 2
203294_s_at	1,8703344	Down	LMAN1	Lectin
214336_s_at	1,885618	Down	COPA	Coatomer protein complex
214975_s_at	1,9469228	Down	MTMR1	Myotubularin related protein 1
212797_at	1,983121	Down	*SORT1*	Sortilin 1
221618_s_at	2,0026515	Down	LOC728198///TAF9B	TAF9B RNA polymerase II
206943_at	2,0033834	Down	*TGFBR1*	Transforming growth factor
205655_at	2,0270846	Down	*MDM4*	Mdm4 p53 binding protein homolog (mouse)
213606_s_at	2,1147816	Down	ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha
206579_at	2,1255388	Down	ZNF192	Zinc finger protein 192
219927_at	2,2972803	Down	*FCF1*	FCF1 small subunit (SSU) processome component homolog (*S. Cerevisiae*)
212185_x_at	1,5050669	Up	MT2A	Metallothionein 2A
212599_at	1,5079852	Up	AUTS2	Autism susceptibility candidate 2
221478_at	1,5125571	Up	BNIP3L	BCL2/adenovirus E1B 19kda interacting protein 3-like
202458_at	1,5177377	Up	PRSS23	Protease
209795_at	1,5322977	Up	CD69	CD69 molecule
206494_s_at	1,5378845	Up	ITGA2B	Integrin
214978_s_at	1,5383469	Up	PPFIA4	Protein tyrosine phosphatase
205927_s_at	1,5472317	Up	CTSE	Cathepsin E
222024_s_at	1,5497096	Up	*AKAP13*	A kinase (PRKA) anchor protein 13
213506_at	1,550675	Up	F2RL1	Coagulation factor II (thrombin) receptor-like 1
220560_at	1,5588487	Up	C11orf21	Chromosome 11 open reading frame 21
213975_s_at	1,5607862	Up	*LYZ*	Lysozyme (renal amyloidosis)
212614_at	1,5785391	Up	ARID5B	AT rich interactive domain 5B (MRF1-like)
207463_x_at	1,5802315	Up	PRSS3	Protease
203695_s_at	1,5820975	Up	DFNA5	Deafness
200872_at	1,5952313	Up	S100A10	S100 calcium binding protein A10
219403_s_at	1,629079	Up	HPSE	Heparanase
208792_s_at	1,6501576	Up	CLU	Clusterin
213438_at	1,6593443	Up	**NFASC**	**Neurofascin homolog (chicken)**
215235_at	1,6661867	Up	SPTAN1	Spectrin
219059_s_at	1,675863	Up	*LYVE1*	Lymphatic vessel endothelial hyaluronan receptor 1
205626_s_at	1,6777687	Up	CALB1	Calbindin 1
221211_s_at	1,7345427	Up	*C21orf7*	Chromosome 21 open reading frame 7
200762_at	1,7381908	Up	DPYSL2	Dihydropyrimidinase-like 2
219476_at	1,7981561	Up	*C1orf116*	Chromosome 1 open reading frame 116
215395_x_at	1,808629	Up	LOC100134294///TRY6	Trypsinogen C///hypothetical protein LOC100134294
219410_at	1,8366171	Up	TMEM45A	Transmembrane protein 45A
205402_x_at	1,8558791	Up	PRSS2	Protease
208966_x_at	1,859521	Up	IFI16	Interferon
212063_at	1,9257536	Up	CD44	CD44 molecule (Indian blood group)
202237_at	2,2827954	Up	NNMT	Nicotinamide N-methyltransferase

Deregulated mRNAs containing conserved *miR-10a* binding sites are depicted in bold. Deregulated mRNAs containing poorly conserved *miR-10a* binding sites are depicted in italics.