Integrative nucleophosmin mutation-associated microRNA and gene expression pattern analysis identifies novel microRNA - target gene interactions in acute myeloid leukemia

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

MicroRNAs are regulators of gene expression, which act mainly by decreasing mRNA levels of their multiple targets. Deregulated microRNA expression has been shown for acute myeloid leukemia, a disease also characterized by altered gene expression associated with distinct genomic aberrations such as nucleophosmin (*NPM1*) mutations. To shed further light on the role of deregulated microRNA and gene expression in cytogenetically normal acute myeloid leukemia with *NPM1* mutation we performed an integrative analysis of microRNA and mRNA expression data sets.

Design and Methods

Both microRNA and gene expression profiles were investigated in samples from a cohort of adult cytogenetically normal acute myeloid leukemia patients (n=43; median age 46 years, range 23-60 years) with known *NPM1* mutation status (n=23 mutated, n=20 wild-type) and the data were integratively analyzed. Putative microRNA-mRNA interactions were validated by quantitative reverse transcriptase polymerase chain reaction, western blotting and luciferase reporter assays. For selected microRNAs, sensitivity of microRNA-overexpressing cells to cytarabine treatment was tested by FACS viability and cell proliferation assays.

Results

Our integrative approach of analyzing both microRNA- and gene expression profiles in parallel resulted in a refined list of putative target genes affected by *NPM1* mutation-associated microRNA deregulation. Of 177 putative microRNA – target mRNA interactions we identified and validated 77 novel candidates with known or potential involvement in leukemogenesis, such as *IRF2*-miR-20a, *KIT*-miR-20a and *MN1*-miR-15a. Furthermore, our data showed that deregulated expression of tumor suppressor microRNAs, such as miR-29a and miR-30c, might contribute to sensitivity to cytarabine, which is observed in *NPM1* mutated acute myeloid leukemia.

Conclusions

Overall, our observations highlight that integrative data analysis approaches can improve insights into leukemia biology, and lead to the identification of novel microRNA - target gene interactions of potential relevance for acute myeloid leukemia treatment.

Key words: microRNA, miRNA, gene expression profiling, GEP, acute myeloid leukemia, AML, *NPM1* mutation.

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Introduction

In recent years, many chromosomal aberrations and/or gene mutations have been identified which alter normal gene function or expression, contribute to leukemic transformation and provide important diagnostic and prognostic information in acute myeloid leukemia (AML).[1] About 40-50% of all cases of AML do not show chromosomal aberrations, and are therefore termed cytogenetically normal (CN-AML). Here, the identification of *NPM1*, *FLT3* or *CEBPA* gene mutations allows the dissection of CN-AML into subgroups with different prognoses.[2] However, the mechanisms of gene deregulation, which is commonly observed in AML, as well as the function of most of the leukemia-associated mutations, including the most common one affecting *NPM1*, are still not fully understood.[1]

NPM1 is involved in various cellular processes, and is important in the regulation of cell proliferation and apoptosis.[3] Mutations in the *NPM1* gene (*NPM1*^{mut}) were found to be the most prevalent genetic alteration in adult AML patients. Of note, 48-64% of CN-AML patients show mutations in *NPM1*,[2,4-5] which result in mutant proteins that are aberrantly localized to the cytoplasm of leukemic cells and thought to be critical for leukemogenesis.[3] AML with *NPM1*^{mut} is generally associated with a good response to induction chemotherapy.[4-6] However, the mechanisms underlying this chemosensitivity are still unknown. Moreover, AML with *NPM1*^{mut} shows a distinct gene expression profile (GEP),[7-9] as well as a distinct microRNA signature,[9-11] which both require further exploration to assess their impact on *NPM1*^{mut} AML pathogenesis.

MicroRNAs (miRNAs) are non-coding RNA molecules of approximately 22 nucleotides, and function as post-transcriptional regulators of gene expression.[12] There is recent evidence showing that mammalian miRNAs predominantly act by mRNA destabilization and subsequent decrease of target mRNA levels.[13] Given the fact that a miRNA can target the mRNA of hundreds of genes,[14-15] this might explain their widespread function in cellular processes such as differentiation, proliferation and apoptosis. Therefore, miRNAs can function both as oncogenes and tumor suppressors, and deregulated expression has been associated with various human cancers, including AML.[16-17] Early studies interrelating miRNA expression and gene expression profiles already revealed interesting insights into altered gene regulation in AML, for example miR-29b mediated *MCL1* regulation.[18] However, large-scale integrative analyses investigating the direct impact of miRNA deregulation on gene expression have not been performed so far. In the current study, we therefore screened miRNA- and gene expression in a defined cohort of adults with CN-AML and focused our efforts on *NPM1*^{mut}-associated miRNA- and gene expression changes. We then investigated the interrelation of miRNA- and gene expression signatures by an integrative analysis of the respective *NPM1*^{mut}-associated profiles.

Design and Methods

The patients' samples and cell lines used are described in the *Online Supplementary Appendix*, which also provides information and data concerning cell culture, miRNA expression profiling, northern blotting and quantitative reverse-transcriptase PCR (qRT-PCR) analysis for miRNA detection, gene expression profiling, data analysis of microarrays, immunoblot analysis, luciferase reporter assays and nucleofection of myeloid cells.

MicroRNA expression profiling

To screen miRNA expression in AML and leukemia cell lines, we set up a microarray platform using a commercially available oligonucleotide probe set based on version 6.0 of the Sanger miRNA database (*mir*Vana miRNA Probe Set, Ambion). Further details on miRNA expression profiling, data analysis and validation are provided in the *Online Supplementary Appendix*.

Transfection with synthetic microRNAs

HeLa cells were transfected with the indicated microRNA mimics (Pre-miRs, Ambion) or a negative control RNA (Pre-miR Negative Control #1, Ambion) at a final concentration of 30 nM using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. Cells were harvested 24 or 48 h post-transfection. For target gene investigation and functional analyses, HEL and K-562 cells were transfected with pre-miR at a final concentration of 100 nM using the Nucleofector II Device and Cell Line Nucleofector Kit V (Lonza; see *Online Supplementary Appendix* for transfection efficiencies, monitored by GFP plasmid transfection).

Quantitative reverse transcriptase polymerase chain reaction for mRNA detection

Total RNA of transfected cell lines was extracted using the *mir*Vana miRNA Isolation Kit (Ambion), and cDNA synthesis using the SuperScript III First-Strand Synthesis System (Invitrogen) was primed with random hexamer primers according to the manufacturer's protocol. Analyses were carried out using the Fast SYBR Green Master Mix (Applied Biosystems) with a 7900HT Fast Real-Time PCR System (Applied Biosystems) in the fast mode. Primer sequences, the cycling program for the genes analyzed (n=47) and primer testing are presented in the *Online Supplementary Appendix* (*Online Supplementary Table S3, Figures S1 and S2*). Samples were assayed in duplicate and three different housekeeping genes (*ACTB, LMNB1, PGK1*) were analyzed for data normalization. Quantity mean values for gene expression were calculated according to standard curves, for which dilution series of cDNA of untransfected HeLa or HEL cells were used. Data were analyzed using SDS 2.3 software (Applied Biosystems) and visualized using GraphPad Prism 4 (GraphPad Software).

Cell proliferation assays and flow cytometric detection of apoptosis

Two hours post-transfection with pre-miRs, cells were either left untreated or treated with a subtoxic concentration of cytarabine [according to previously measured dose-response curves: ~80% viability in untransfected cells (measured 24 h after exposure to the drug); HEL: 0.01 μM cytarabine; K-562: 1 μM]. Cells were diluted 24 h post-transfection 1:5 in medium with or without cytarabine to prevent overgrowth of the culture. Cell proliferation was measured at 48, 72 and 96 h post-transfection using the ATP quantitating CellTiter-Glo Luminescent Cell Viability Assay (Promega). At the same time-points, apoptosis induction was measured by double-staining the cells with annexin-PE/7-AAD (BD Pharmingen) using a FACSCalibur flow cytometer (BD). FACS data were analyzed using CellQuest Pro software (BD Pharmingen).

Statistical analyses

Group-wise comparisons of the distributions of clinical variables or experimental results were performed using Fisher's exact test, one-sample t test or unpaired t test, as appropriate. All tests were two-tailed and an effect was considered statistically significant if

the *P* value was 0.05 or less. Data were visualized and analyzed using GraphPad Prism 4.

Results

Parallel analysis of NPM1 mutation-associated microRNA and gene expression changes in cytogenetically normal acute myeloid leukemia

By profiling miRNA and gene expression in 43 CN-AML samples, we identified characteristic *NPM1*^{mut}-associated expression patterns both at the miRNA and mRNA level. A *NPM1*^{mut}-associated gene expression signature based on our data (comprising 730 genes down-regulated in *NPM1*^{mut} cases and 663 up-regulated genes) was in agreement with published signatures,[7-9] showing for example the characteristic up-regulation of *HOX* genes and an inverse correlation with *MN1* and *BAALC* (*Online Supplementary Table S4*). With regard to the miRNA profiling, 66 miRNAs were differentially expressed in *NPM1*^{mut} compared to *NPM1* wild-type (*NPM1*^{wt}) cases (Figure 1; *Online Supplementary Table S5*). These miRNAs comprised, among others, the oncogenic miR-17-92 cluster and miR-155, as well as miRNAs with a known tumor-suppressor role such as the members of the let-7 family, the miR-15/16 cluster, and the miR-29 family. The vast majority of the miRNAs in this signature was up-regulated in *NPM1*^{mut} CN-AML and showed a broad overlap with previously reported *NPM1*^{mut} miRNA signatures,[9-11] based on which we defined a "core set" of 33 up-regulated miRNAs (core miRNAs defined by overlapping results of our supervised analyses and the miRNA signature published by Garzon *et al.*[10]: see Figure 1 and *Online Supplementary Table S5*). Consistently, hierarchical clustering based on the supervised findings was *NPM1*-associated (P=0.0238; Figure 1), although in line with the findings of Garzon *et al.*, we did not observe a clear-cut separation of *NPM1*^{mut} and *NPM1*^{wt} cases, which might be due to additional yet unknown secondary genomic events. For exemplary miRNAs, microarray findings were validated by northern blot analysis and/or qRT-PCR (*Online Supplementary Figure S3*).

In order to identify suitable cell lines for future functional analyses with the aim of over-expressing the miRNAs highly expressed in *NPM1*^{mut} AML, we profiled miRNA expression of 14 *NPM1*^{wt} leukemia cell lines. We identified nine cell lines that co-clustered with the NPM1^{wt}-predominant subgroup (Figure 1), which could serve as potential models for functional analyses.

Integrative analysis of NPM1 mutation-associated microRNA and gene expression signatures

To identify putative target genes of *NPM1*^{mut}-associated miRNAs, an integrative analysis of miRNA expression and gene expression data was performed (Figure 2). First, we generated target gene lists for the core set of 33 up-regulated miRNAs by using the miRGator database,[19] which can combine three different prediction tools [MicroCosm Targets (formerly miRBase Targets, M), TargetScan (T) and PicTar (P)] which are based on different algorithms and prediction stringencies.[12] We included all genes that were predicted by any one of these algorithms to obtain a gene list comprising all potential targets, which resulted in 8293 unique predicted target genes (Figure 2B). The predictions by TargetScan and PicTar have a high degree of overlap because they require more stringent seed region pairing than MicroCosm Targets, and only few targets were predicted by all three algorithms (see *Online Supplementary Table S6*; candidates are marked M/T/P according to the respective algorithm by which they were predicted). To further refine this theoretical list (constituting a theoretical *NPM1*^{mut}-associated GEP) of potential direct miRNA targets in *NPM1*^{mut} AML, we filtered for genes down-regulated in *NPM1*^{mut} *versus* *NPM1*^{wt} cases, as a recent study showed that mammalian miRNAs predominantly act to decrease target mRNA levels.[13] Thus, we correlated *NPM1*^{mut}-associated miRNA and mRNA expression, both obtained from the same patients to narrow down putative target genes of which mRNA levels might have been directly affected by the respective miRNAs. As many factors can have an impact on gene expression levels, such an approach can only reveal direct targets in which no influencing factors obscure the direct miRNA-mRNA interaction, and in addition will likely reveal many false positive interactions. Nevertheless, we hypothesized that such an approach might allow us to identify and validate novel miRNA-mRNA interaction partners, although the potential direct miRNA targets were not statistically significantly enriched in the *NPM1*^{mut}-associated mRNA signature, most likely due to fact that gene expression is influenced by many factors. For 307 *NPM1*^{mut}-associated down-regulated genes the putative targeting miRNA was up-regulated (genes highlighted in *Online Supplementary Table S4*; core miRNAs predicted to target the respective genes are listed in *Online Supplementary Table S7*). These included candidate genes implicated in tumorigenesis and/or leukemogenesis, such as *APP*, *CCND1*, *IRF2*, *SPARC*, *MN1*, *SERPINB9* and *KIT*. Interestingly, many of these genes are also putative targets of not only one, but several miRNAs of the *NPM1*^{mut}-associated miRNA signature, and/or have several binding sites for the respective individual miRNAs.

Validation of target genes by quantitative reverse transcriptase polymerase chain reaction in cells transfected with synthetic microRNAs

To further investigate individual miRNA-target gene relations, we first studied the impact of transfection of synthetic miRNA mimics on target gene mRNA levels by qRT-PCR. As testing all 33 miRNAs and 307 putative direct target genes was not feasible, we focused on 11 miRNAs representative of most miRNA families present in the *NPM1*^{mut} signature and selected 42 genes of potential relevance for leukemogenesis and/or tumorigenesis (n=177 different miRNA-target gene pairs predicted by at least one of the three prediction algorithms M/T/P, see *Online Supplementary Table S6*). Expression of the putative target genes was analyzed 24 and 48 h post-transfection (exemplary genes are depicted in Figure 3; detailed overview of results in *Online Supplementary Table S6*). For data normalization three different housekeeping genes (*ACTB*, *LMNB1*, *PGK1*) were utilized as endogenous controls (Figure 3), because housekeeping genes themselves can be targeted by miRNAs (*Online Supplementary Table S6*). For example, normalization of the measurements for *SPARC* and *IRF2* using *LMNB1*, known to be targeted by miR-23a,[20] might lead to misinterpretation of results if compared to normalization with the other housekeeping genes (Figure 3, open symbols; *Online Supplementary Table S8*).

As expected, target gene validation was successful for known miRNA-target gene pairs (Figure 3; *Online*

Supplementary Table S6, blue shaded boxes), which were almost all detected by our qRT-PCR screen (with two exceptions out of 11), thereby validating our experimental approach of monitoring mRNA changes to identify novel miRNA-target gene interactions. Examples include *E2F1* regulated by miR-20a,[21] *SPARC* targeted by miR-29a,[22] and *CCND1* regulated by miR-15a and miR-20a.[23-24] In addition, many novel pairs were identified, including *SPARC*, which was also significantly affected by miR-23a, and the cell cycle regulator *CCND1*, which was influenced by several miRNAs of the signature. Further novel target genes included interferon regulatory factor-2 (*IRF2*), regulated by miR-20a and miR-23a, the receptor tyrosine kinase *KIT* (regulated by miR-19a and miR-20a) and *SERPINB9* (regulated by let-7a, miR-29a and miR-369-3p). In total, 35 of the 177 miRNA-target gene relations (19.8%) showed a mRNA level reduction of more than 30% after miRNA transfection, a cut-off defined by effects that in general were highly significant ($P<0.001$; *Online Supplementary Table S6*, green shading, and *Online Supplementary Figure S4*), and 42 of 177 relations (23.7%) showed a weaker but consistent and significant mRNA reduction in the range of 10-30% ($P<0.05$; *Online Supplementary Table S6*, yellow shading). Thus, in total we observed for 43.5% of miRNA-target gene pairs (77 of 177) an effect of miRNA transfection on mRNA levels.

Furthermore, selected miRNA-target gene interactions (n=13) were validated in two different myeloid cell lines (HEL and K-562; *Online Supplementary Figure S5*).

Validation of targets on protein level and by luciferase reporter experiments

Following qRT-PCR validation of miRNA-target gene pairs, we looked at protein expression of selected candidates in HeLa cells transfected with miRNA mimics. Western blot analysis of targets showed that the transfected

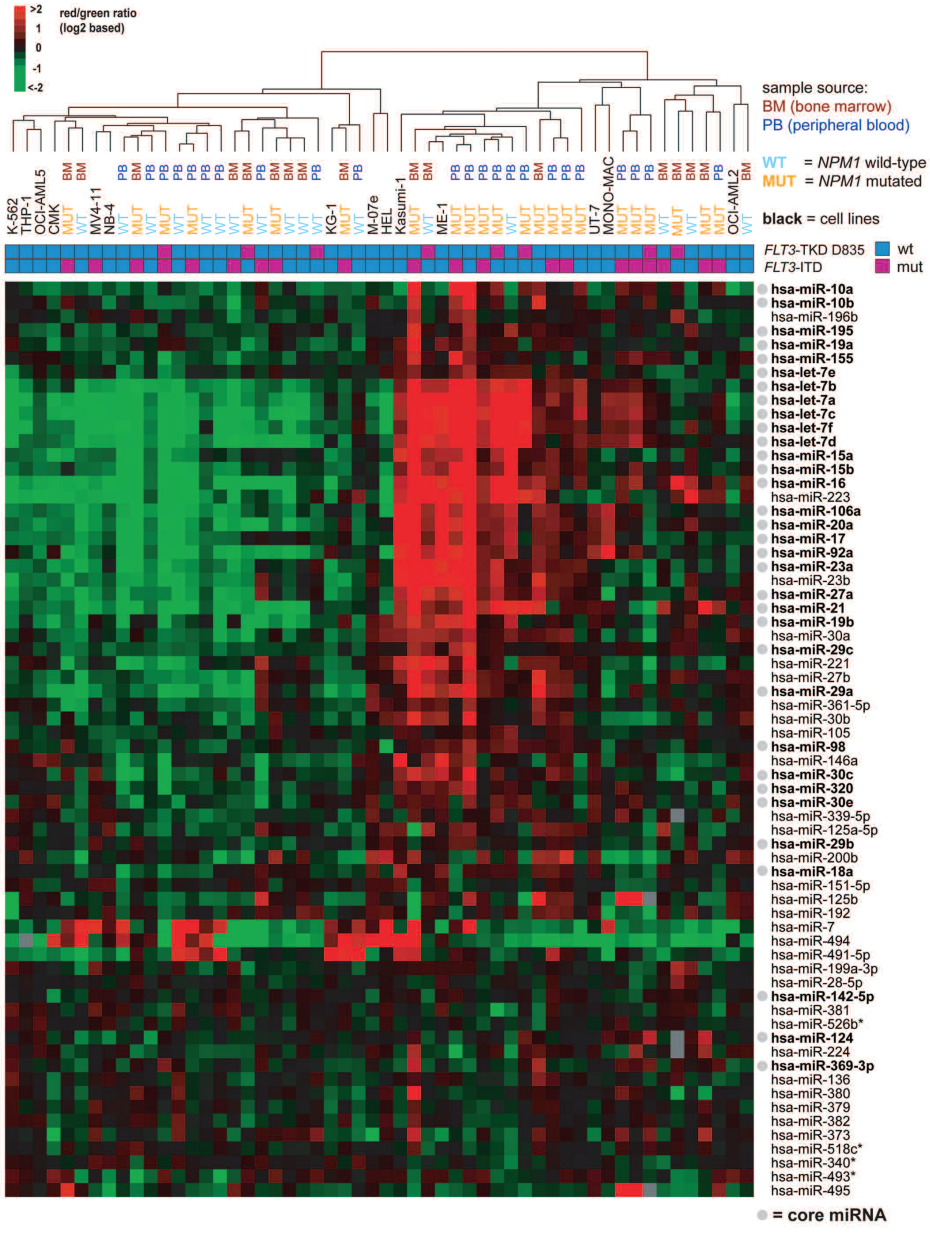

Figure 1. Hierarchical clustering based on *NPM1*^{mut}-associated miRNAs as determined by SAM analysis. This heatmap displays expression levels of the miRNAs associated with *NPM1*^{mut} CN-AML (determined by SAM *NPM1*^{mut} *versus* *NPM1*^{wt}; false discovery rate = 0.09631). Sixty-six miRNAs (rows) and 40 AML patients with normal karyotype (23 of these with *NPM1*^{mut}) and 14 *NPM1*^{wt} leukemia cell lines (columns) were hierarchically clustered (arrays from 3 patients were excluded due to borderline quality). Mean-centered $\log_2$ expression ratios are depicted by the color scale, as indicated. Samples have been color coded according to the underlying molecular aberration and sample source (bone marrow or peripheral blood). MUT = *NPM1* mutation; WT = *NPM1* wild-type; *FLT3*-ITD = internal tandem duplication; TKD D835 = tyrosine kinase domain (TKD) mutation at codon D835. Cell line names are shown in black. *NPM1*^{mut} and *NPM1*^{wt} patients are significantly differentially distributed between the two groups (P=0.0238; Fisher's exact test), whereas there is no significant difference for *FLT3*^{mut} and *FLT3*^{wt} samples (P=0.2756). Sample source (bone marrow *versus* peripheral blood) did not result in significant miRNA expression pattern differences (P=0.7496). The core 33 upregulated miRNAs used for the subsequent integrated analysis are printed in bold and marked with a gray dot.

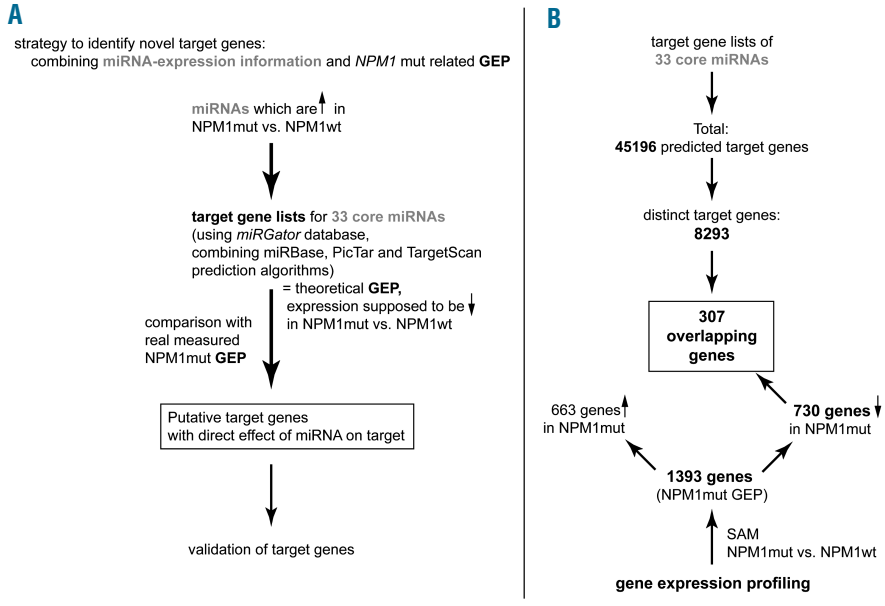

Figure 2. Integrative data analysis strategy and results. (A) Strategy description of integrative analysis of miRNA- and gene-expression data. (B) Results of integrative analysis. Target gene lists of 33 core miRNAs resulted in a total of 45196 target genes (multiple entries per gene, because of being predicted targets by several miRNAs with several target sites), which represent 8293 distinct gene names).

miRNAs also led to a down-regulation of the respective target protein levels (compared to negative control RNA transfected cells), such as the positive controls E2F1, targeted by miR-20a and miR-106a,[21] and NRAS, targeted by let-7a (Figures 4A-B).[25] APP protein levels were reduced by miR-20a and miR-106a transfection, as previously reported.[26-27] As seen in the qRT-PCR analyses, cyclin-D1 levels were reduced by several miRNAs of the signature, here shown for miR-20a (48 h post-transfection, 22% reduction compared to negative control),[24] and the novel regulators miR-106a, let-7a (27% and 34% reduction at 48 h, and 35% and 54% reduction at 72 h, respectively) and miR-155 (44% reduction at 48 h). Moreover, the newly identified miR-20a target IRF2 was reduced on protein level, supporting the qRT-PCR results. As previously described, SPARC levels were strongly down-regulated by miR-29a (46% reduction),[22] and also, to a lesser extent (9%), by miR-23a, again as seen in the qRT-PCR analysis.

To further validate the direct regulatory effect of miRNAs of the signature on their target genes, we performed luciferase reporter assays. Compared to negative control-transfected cells, the firefly/renilla luciferase ratio was strongly reduced in the miRNA-transfected cells for the positive control *SPARC* plus miR-29a (58% reduction). A significant reduction was also apparent for the newly identified miRNA-target gene pairs *CLCN3*–miR-15a, *CRKL*–miR-15a, *IRF2*-miR-20a, *KIT*–miR-19a and -miR-20a, *MN1*–miR-15a as well as *SERPINB9*-miR-29a (29-50% reduction, $P<0.001$; dark gray bars in Figures 4 C-D). Here, the mutant binding sites were able to attenuate repression (Figures 4 C-D, light gray bars and *Online Supplementary Figure S6*), suggesting a direct regulation of the target by the respective miRNA. For 3'UTR with two predicted miRNA binding sites, mutation of both sites was necessary to diminish repression of luciferase effectively (Figure 4D).

NPM1 mutation-associated microRNAs might contribute to chemosensitivity

With our integrative approach we identified a variety of novel miRNA- target gene pairs of our *NPM1*^{mut}-associated miRNA signature, consisting of various miRNAs whose expression was significantly up-regulated in *NPM1*^{mut} compared to *NPM1*^{wt} cases. To analyze the biological role of these miRNAs, we looked at the effects of ectopic miRNA expression in two *NPM1*^{wt} leukemic cell lines (HEL and K-562). As expected, both cell lines expressed the miRNAs of the signature at a low level (Figure 1), and were therefore suitable for studying the effects of miRNA over-expression.

In our experimental set-up, we did not observe a significant growth inhibitory effect for miR-29a, miR-30c or miR-369-3p transfection in HEL and K-562 cells, if the cells were left untreated after transfection and were not exposed to additional stress (Figure 5A). In agreement, there was also no apoptosis induction effect following miRNA over-expression in either cell line (Figure 5B).

Recently, it was shown for breast cancer cells that miRNA expression can increase sensitivity of cells for example to doxorubicin- or VP-16-induced loss of viability.[28-29] Therefore, we next tested for a possible sensitization effect by these miRNAs in our leukemic cell line models. Transfected cells were exposed to subtoxic concentrations of cytarabine, which constitutes the backbone of chemotherapy in AML treatment. Interestingly, after cytarabine treatment a growth inhibitory effect was observed in miR-29a as well as in miR-30c over-expressing cells, but not in miR-369-3p over-expressing cells (Figure 5C). Moreover, in cytarabine-treated cells, a remarkable induction of apoptosis could be observed for miR-29a and miR-30c beginning 48 h post-transfection and treatment start and peaking at 72-96 h post-transfection (87% increase in apoptotic cells by miR-29a and 52% by miR-30c for K-562 cells at 72 h; Figure 5D). For HEL cells, the induction of apoptosis by miR-30c was even more pronounced than that by miR-29a, peaking at 96 h after transfection (56% increase in apoptotic cells by miR-30c and 39% by miR-29a at 96 h). Thus, the over-expression of distinct miRNAs of the *NPM1*^{mut} signature can lead to sensitization to cytarabine.

Discussion

Recently, several miRNA profiling studies in AML revealed an up-regulation of distinct miRNAs in *NPM1*^{mut}

versus NPM1^{wt} CN-AML cases as one of the most consistent signatures in AML.[9-10] In accordance, our profiling approach also revealed a strong *NPM1*^{mut}-associated signature of up-regulated miRNAs, which substantially overlapped with published data and comprised many miRNAs known to be implicated in tumorigenesis and/or leukemogenesis. Exemplary miRNAs with an oncogenic potential included miR-155,[30] as well as miR-17-92 cluster members.[31] Furthermore, our core miRNA signature also comprised tumor-suppressor miRNAs including the miR-29 and miR-30 family. Here, one might speculate that this could in part account for the favorable response to therapy in *NPM1*^{mut} cases, or *vice versa*, low expression of the respective tumor-suppressor miRNAs in *NPM1*^{wt} cases might contribute to an inferior outcome. Therefore, we hypothesize that over-expression of a distinct set of miRNAs might play an important role in *NPM1*^{mut} CN-AML, resulting in deregulated expression of target genes possibly contributing to leukemogenesis. So far, this issue has not been addressed systematically for *NPM1*^{mut} CN-AML.

In order to identify putative target genes of the *NPM1*^{mut}-associated miRNAs, miRNA target gene lists can be generated using prediction algorithms, such as the miRGator database, which usually reveal a large number of candidates. This list could be effectively narrowed down to a set of promising candidate genes potentially implicated in *NPM1*^{mut}-associated leukemogenesis by integrating this theoretical *NPM1*^{mut}-associated target gene list with GEP data. Similar to our approach, other studies in AML have combined miRNA and gene expression information; however, many analyzed only a limited number of miRNAs and the expression of their putative target genes at a time (such as miR-181a and the *HOX*-associated miRNA miR-196a, -10a and -10b).[32-33] Thus, in addition to a recent study by Havelange *et al.*, who integrated miRNA and mRNA expression from 48 AML cases,[34] our study represents an additional large integrative approach that combines miRNA and gene expression data from the same patient cohort, with the purpose to understand the complex regulatory network of miRNA and associated target genes involved in the pathogenesis and clinical characteristics of *NPM1*^{mut} AML.

Recently, it has been shown that mammalian miRNAs act mainly via mRNA-destabilization and subsequent decrease of target mRNA levels and not only, as previously thought, via translational repression.[13,15] We, therefore, evaluated putative miRNA-target gene interactions following over-expression of the respective miRNAs in cell line models by mRNA expression analysis using qRT-PCR, and in contrast to Havelange *et al.* focused less on gene ontology and network analysis.[34] This approach allowed us to screen a comprehensive list of target genes of representatives of the miRNA families deregulated in *NPM1*^{mut} AML.

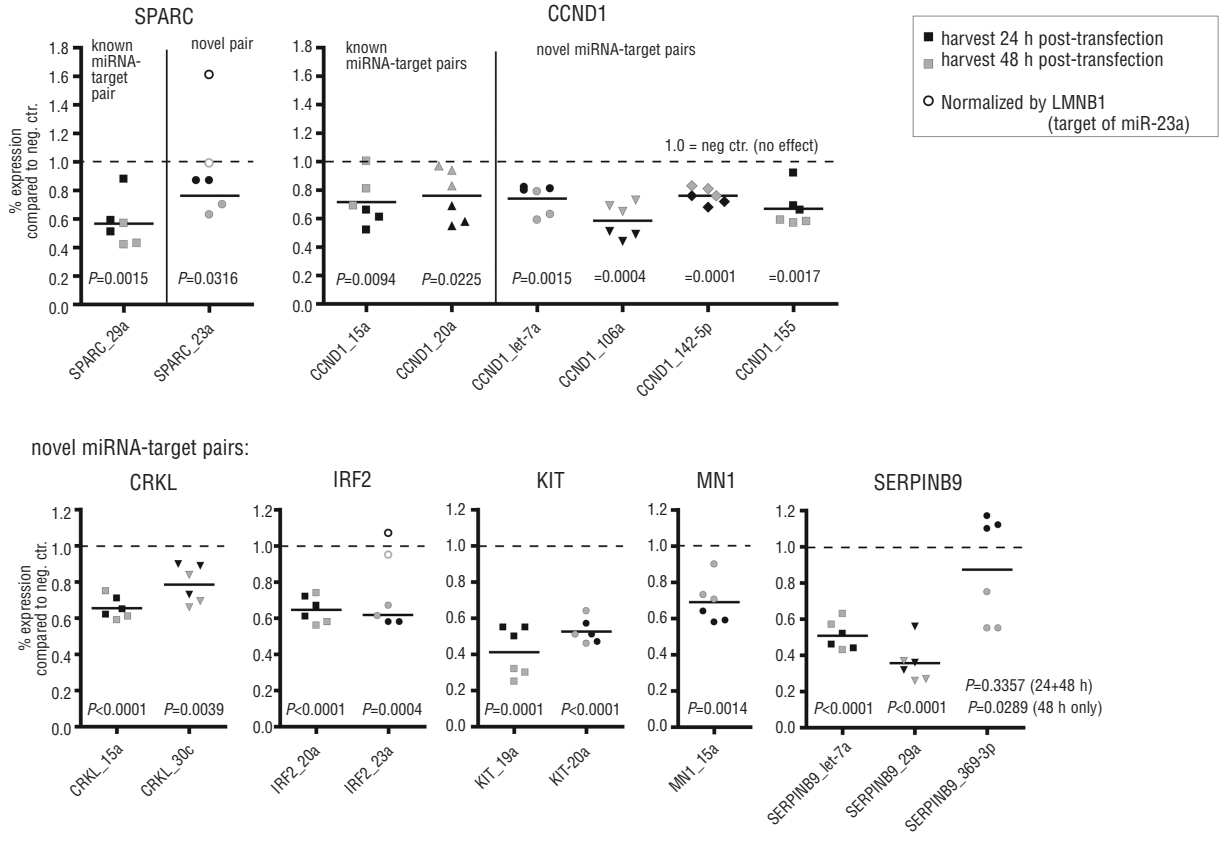

Figure 3. Validation of predicted miRNA targets by qRT-PCR. Normalized qRT-PCR analyses of selected miRNA target genes in HeLa cells which were transfected with the indicated miRNA mimic or negative control and harvested 24 or 48 h post-transfection. Each data point represents the normalization with one of three housekeeping genes (*ACTB*, *LMNB1*, *PGK1*) and is the mean of two independent transfection experiments (with 2 technical replicates each). Each value is calculated as percentage expression of target gene mRNA of miRNA-of-interest transfected cells compared to negative control (1.0 = 100%) transfected cells. The horizontal lines show mean reduction. All effects were tested for significance (mean different from 1; one-sample t test). For *SPARC* and *IRF2*, outliers (open symbols) are the values normalized by *LMNB1* (a known target of miR-23a) and were therefore excluded for calculation of the mean and for statistical testing.

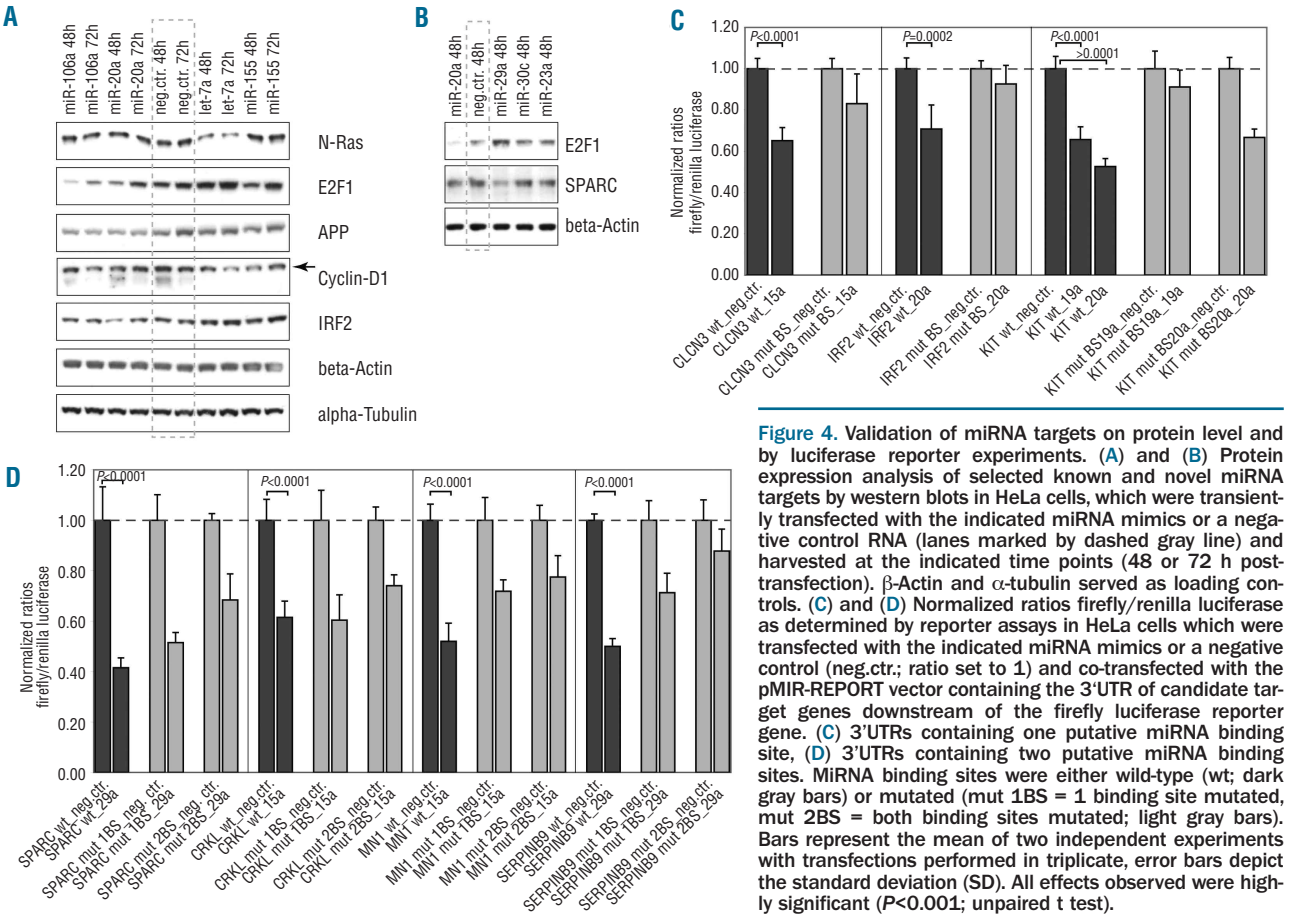

Figure 4. Validation of miRNA targets on protein level and by luciferase reporter experiments. (**A**) and (**B**) Protein expression analysis of selected known and novel miRNA targets by western blots in HeLa cells, which were transiently transfected with the indicated miRNA mimics or a negative control RNA (lanes marked by dashed gray line) and harvested at the indicated time points (48 or 72 h post-transfection). β-Actin and α-tubulin served as loading controls. (**C**) and (**D**) Normalized ratios firefly/renilla luciferase as determined by reporter assays in HeLa cells which were transfected with the indicated miRNA mimics or a negative control (neg.ctr.; ratio set to 1) and co-transfected with the pMIR-REPORT vector containing the 3'UTR of candidate target genes downstream of the firefly luciferase reporter gene. (**C**) 3'UTRs containing one putative miRNA binding site, (**D**) 3'UTRs containing two putative miRNA binding sites. MiRNA binding sites were either wild-type (wt; dark gray bars) or mutated (mut 1BS = 1 binding site mutated, mut 2BS = both binding sites mutated; light gray bars). Bars represent the mean of two independent experiments with transfections performed in triplicate, error bars depict the standard deviation (SD). All effects observed were highly significant (*P*<0.001; unpaired t test).

Members of these miRNA families mostly have the same predicted targets due to identical seed region sequences.[12] We were not only able to validate known miRNA-target gene interactions such as miR-20a-*E2F1* and miR-15a-*CCND1*, but also to identify a variety of novel miRNA targets. Thus, while our mRNA measurement-based approach might miss single target genes, monitoring mRNA levels nevertheless constitutes a feasible and fast method to identify novel miRNA targets, especially as candidates could also be validated by western blotting and luciferase reporter assays.

One example of a newly validated miRNA target is *IRF2*, coding for a transcriptional suppressor of type I interferon (IFN) signaling. Mice deficient for *IRF2* show a substantial decrease of hematopoietic stem cells (HSCs) and a significant increase of immature progenitor cells.[35] Being essential for the preservation of the HSC pool, IRF2 normally suppresses IFN signaling in wild-type HSCs, thereby maintaining HSCs mostly in a quiescent state. In accordance, IFN-α stimulates the proliferation and turnover of dormant HSCs *in vivo*, and the cycling HSCs then become sensitive to antiproliferative chemotherapeutic agents, which are not effective for dormant HSCs.[35-36] This mechanism might in part contribute to the chemosensitivity of *NPM1*^{mut} AML cases. Also, *IRF2* knock-down associated growth inhibition and induction of differentiation in a leukemia cell line further emphasizes the role of *IRF2* deregulation in leukemogenesis.[37] Moreover, the receptor tyrosine kinase KIT, important for normal hematopoiesis,[38] was validated as a target of several *NPM1*^{mut}-associated miRNAs. KIT is crucial for maintaining HSC self-renewal and quiescence and was shown to be down-regulated in *IRF2* deficient cells.[35] Similarly, the correlation of low *MN1* expression and *NPM1*^{mut} might be explained by miRNA deregulation, and in line with our findings a recent *MN1*-associated miRNA signature showed a negative correlation of *MN1* expression and the miR-15/16 cluster.[39] Functioning as an oncogenic transcriptional co-activator, high expression of *MN1* is associated with poor outcome in CN-AML, and especially with a poor response to induction chemotherapy.[40] In accordance with this, there is recent evidence from a murine AML model showing that *MN1* over-expression leads to resistance to cytarabine and doxorubicin.[41] Thus, the down-regulation of genes involved in chemosensitivity such as *MN1*, *KIT* and *IRF2* by *NPM1*^{mut}-associated miRNAs might be linked to the favorable response to chemotherapy observed for *NPM1*^{mut} AML.

In addition, several studies have shown that miRNAs can predict sensitivity or resistance to anticancer-treatment: for example, miR-30c down-regulation has been associated with drug-resistant ovarian cancer cells, and miRNAs were also reported to influence sensitivity to chemotherapy.[29] In accordance with these findings, our analysis of the biological role of selected up-regulated *NPM1*^{mut}-associated miRNAs showed that over-expression of miR-29a and miR-30c can sensitize cells to cytarabine, leading to growth inhi-

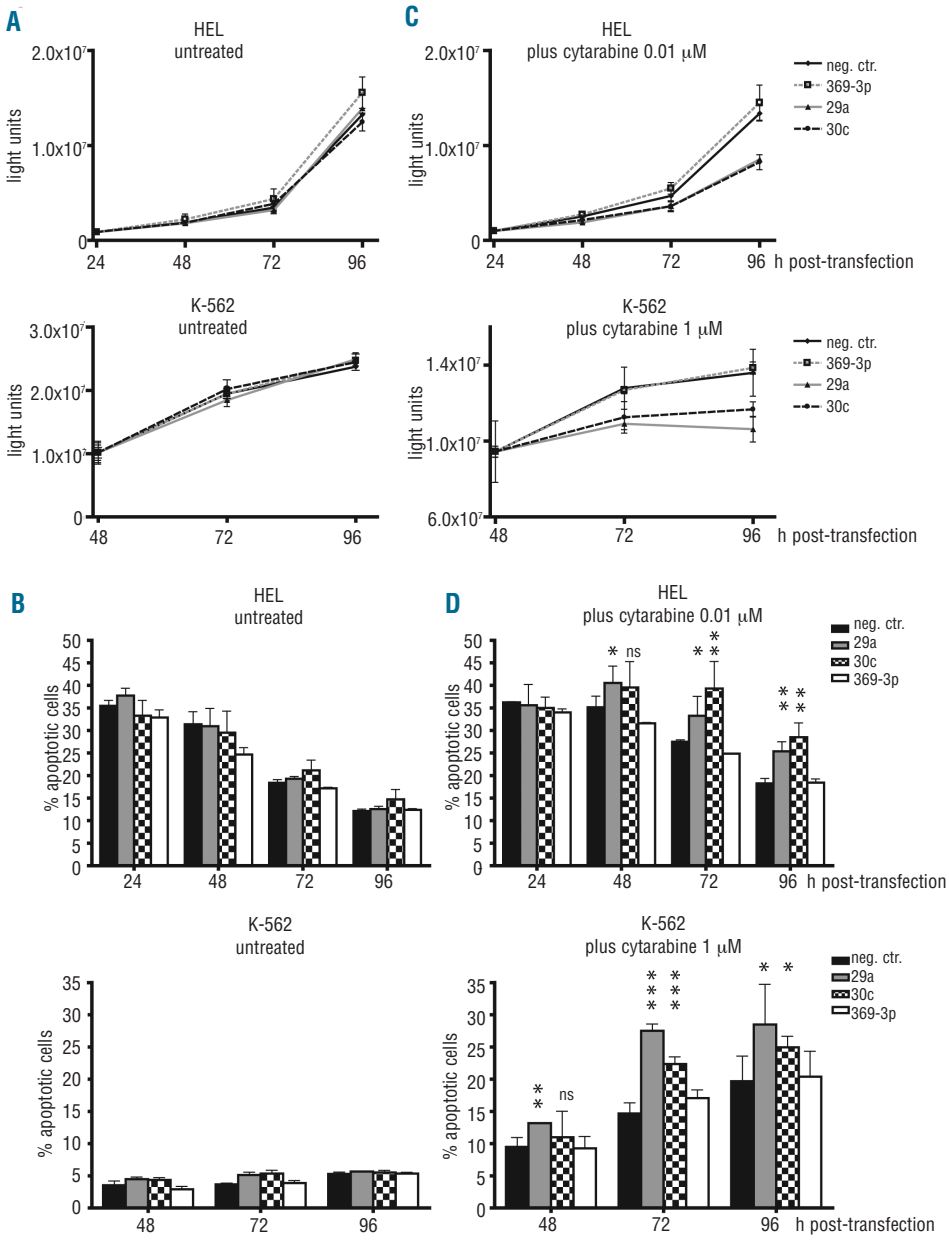

Figure 5. Functional effect of over-expression of miRNAs of the *NPM1*^{mut} signature. (A) and (C) Growth curves of HEL cells (upper panels) or K-562 cells (lower panels) transfected with miRNA mimics of miR-29a, -30c, -369-3p or a negative control. Cells were either left untreated (A) or were exposed to subtoxic concentrations of cytarabine (C) after transfection (HEL: 0.01 μM cytarabine; K-562: 1 μM cytarabine). Cell growth was monitored by CellTiter-Glo assay at the indicated time-points post-transfection. (B) and (D) Monitoring of apoptosis induction by double-staining of cells with annexin-PE/7-AAD at the indicated time-points post-transfection. Cells were either left untreated (B) or were exposed to subtoxic concentrations of cytarabine (D), as mentioned above. The results (mean of 3 independent experiments; error bars show standard deviation) are shown as percentage of apoptotic cells (annexin-PE positive plus annexin-PE/7-AAD double-positive cells) and were tested for significance (compared to neg.ctr.).

bition and apoptosis induction following low-dose cytarabine treatment. This tumor-suppressive role for the miR-29 family has also been shown in a xenograft leukemia model,[18] and our results in cell lines indicate that over-expression of miR-30c has a similarly pronounced growth inhibitory and apoptosis-inducing effect as miR-29a. Thus, the over-expression of tumor-suppressive miRNAs in *NPM1*^{mut} AML, in addition to the influence of miRNAs on single genes as mentioned above, might contribute to the favorable response to chemotherapy. On the other hand, down-regulation of the miR-29 and miR-30 family members in *NPM1*^{wt} cases might play an important role in leukemogenesis. Furthermore, a recent study inferring miRNA networks from miRNA expression data in normal tissue or cancer also suggested a link between the miR-29 and miR-30 family.[42] In normal tissue as well as in AML, the expression patterns of miR-29 and miR-30 family members are closely related, thereby implying co-regulation and a potential collaborative tumor-suppressor mechanism. Therefore, a miRNA-based therapy using miR-30c, as already suggested for the miR-29 family,[18] and its ability to induce chemosensitivity, might be an interesting approach for the treatment of AML.

In conclusion, our integrative data analysis approach combining miRNA and gene expression information led to the identification of a valuable panel of novel miRNA-target gene interactions that will benefit future investigations of the biology of *NPM1*^{mut} AML. Further studies will be necessary to explore the functional impact of the respective interconnections in more depth and should also focus on the mechanisms underlying the miRNA deregulation. Nevertheless, the chemosensitivity-associated candidates identified in our study may contribute to an improved understanding of chemoresistance in *NPM1*^{wt} cases and AML in general and ultimately result in novel therapeutic strategies for adverse-risk AML.

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