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Functional implications of microRNAs in acute myeloid leukemia by integrating microRNA and mRNA expression profiling

Violaine Havelange[1,2,3,4], **Nicole Stauffer**[2,3], **Catherine C.E. Heaphy**[2,3], **Stefano Volinia**[1,3], **Michael Andreeff**[5], **Guido Marcucci**[1,2,3], **Carlo M. Croce**[1,3], and **Ramiro Garzon**[2,3]

[1]Department of Molecular Virology, Immunology and Medical Genetics, The Ohio State University, Columbus,OH, USA

[2]Division of Hematology, The Ohio State University, Columbus, OH, USA

[3]The Comprehensive Cancer Center, The Ohio State University, Columbus, OH, USA

[4]Hematological Section, The Human Genetic Center, Universite´ Catholique de Louvain, Brussels, Belgium

[5]Section of Molecular Hematology and Therapy, Department of Blood and Bone Marrow Transplantation, The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA

Abstract

Background—MicroRNAs expression is deregulated in acute myeloid leukemia, but the corresponding functional microRNA-controlled pathways are poorly understood. Integration of mRNA and microRNA expression profiling may allow the identification of functional links between the whole transcriptome and microRNome that are involved in myeloid leukemogenesis.

Methods—Therefore, here we integrated microRNA and mRNA expression profiles obtained from 48 newly diagnosed acute myeloid leukemia patients by using two different microarray platforms and performed correlation, gene ontology and network analysis. Experimental validation was also performed in acute myeloid leukemia cell lines using microRNA mimics oligonucleotides and functional assays.

Results—Our analysis identified a strong positive correlation of HOX related genes with *miR-10* and *miR-20a*. Furthermore, we observed a negative correlation between *miR-181a* and *-181b*, *-155* and *-146* expression with that of genes involved in immunity and inflammation (e.g. *IRF7* and *TLR4*) and a positive correlation between *miR-23a*, *miR-26a, miR-128a* and *miR-145* expression with that of pro-apoptotic genes (e.g., *BIM* and *PTEN*). These correlations were confirmed by gene ontology analyses, which evidenced the enrichment of members of the homeobox, immunity and inflammation and apoptosis biologic process, respectively. Furthermore, we validated experimentally the association of *miR-145, miR-26a* and *miR-128a* with apoptosis in acute myeloid leukemia.

Conclusions—Our results indicate that by integrating the transcriptome and microRNome in acute myeloid leukemia cells is possible to identify previously unidentified putative functional microRNA-mRNA interactions in acute myeloid leukemia.

Keywords

microRNA; networks; AML; microarrays

Correspondence: Ramiro Garzon at The Ohio State University, Comprehensive Cancer Center, BRT building, Room 1084, 460 12th Ave, Columbus, OH (USA) 43210 or at ramiro.garzon@osumc.edu.

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Introduction

MicroRNAs (miRNAs) are small non-coding RNAs of 18–25 nucleotides in length that regulate gene expression by repressing translation and/or accelerating mRNA decay.[1] MiRNAs are involved in critical biological processes, including development, cell differentiation, apoptosis, proliferation and hematopoiesis.[2–5] Previous data indicate that miRNAs are deregulated in diseases, such as dementia, heart disease and cancer.[6–8] Recently, five large scale studies reported distinctive miRNA expression signatures associated with cytogenetics and molecular abnormalities and outcome in acute myeloid leukemia (AML).[9–15] Up-regulation of *miR-155* and *miR-10a* and *-10b* have been described in cytogenetically normal (CN)–AML with *FLT3-ITD* and nucleophosmin mutations respectively.[9–10] Over-expression of *miR-181* family members has been found up-regulated in CN-AML with *CEBPA* mutations and associated with good outcome in high risk CN-AML, independent from *FLT3-ITD* or *CEBPA* mutation status.[11–12] Concerning recurrent chromosomal abnormalities, translocations involving the mixed lineage leukemia (*MLL*) gene in 11q23, are associated with a distinctive miRNA signature that includes up-regulation of *miR-191* and down-regulation of *miR-29* family members.[9] Likewise, up-regulation of *miR-126* have been described in patients with core binding factor leukemias, such as t(8;21) and inv16 and in those with CN-AML over-expressing *MN1*, which impact negatively on outcome.[13–14,16]

Despite this progress, the functions of most of the deregulated miRNAs in AML are currently unknown. A critical step in that direction is to identify the target genes and pathways that are deregulated by miRNAs during leukemogenesis. However, this has been proven to be a difficult task, since miRNAs are predicted to control ~20–30% of the human genome and a single miRNA can regulate a large number of target genes and multiple miRNAs may regulate the same target.[1] Recently, a novel approach has been developed to identify pathways regulated by miRNAs by integrating miRNA with mRNA expression profiles obtained using high–throughput platforms, such as microarrays.[17–21] Nielsen et al, integrated miRNA and mRNA expression in neuronal rat progenitors during embryonic differentiation and identify regulatory networks underlying the onset of cortical neurogenesis.[17] Liu et al analyzed miRNA and mRNA expression profiles obtained using qRT-PCR and microarrays respectively, from 12 human brain tumors biopsies and inferred and validated a tumor suppressor pathway linked to *miR-181c*.[18]

Therefore, to discover relevant functional miRNA-mRNA relationships in AML, here we integrate miRNA and mRNA expression profiles obtained from newly diagnosed AML patients by using two different microarray platforms and performed correlation, gene ontology and network analysis. The integration of a whole genome approach including non–coding RNAs may lead to an improved understanding of AML biology and identified novel targets for treatment.

Design and Methods

Patient's samples

Frozen diagnostic bone marrow (BM) or peripheral blood (PB) samples were obtained from 48 adult newly diagnosed AML patients from the MD Anderson Tissue bank. Patient's samples were prepared by Ficoll-Hypaque (Nygaard) gradient centrifugation, enriched for leukemic cells by CD3/CD19 depletion using MACS (Miltenyi Biotech, Auburn, CA) and cryopreserved as previously described.[9] Cytogenetic analyses of the samples were performed at diagnosis, using unstimulated short-term (24-, 48-, and 72-hour) cultures with or without a direct method and G-banding. The criteria used to describe a cytogenetic clone

and description of karyotype followed the recommendations of the International System for Human Cytogenetic Nomenclature.[22] At least 20 bone marrow metaphase cells were analyzed in patients designated as having a normal karyotype. *FLT3-ITD*, activation loop *D835* and *NPM1* mutations analysis was performed as previously described.[10] All patients gave inform consent for cryopreservation and use of the samples for molecular studies. Approval was obtained from the institutional board review from the MD Anderson Cancer Center.

Microarray experiments

Total RNA was extracted using tryzol reagent (Invitrogen). The integrity of these total RNAs was assessed using an Agilent 2100 bioanalyzer. Five micrograms of total RNA was hybridized on the custom microarray chip (OSUCCC miRNA microchip version 3.0). This array contains around 1,100 probes (including 345 human and 249 mouse miRNA genes spotted in duplicate). For the gene expression profilings, 2–3 micrograms of RNA was analyzed by Affymetrix U133 plus 2.0 GeneChips (Santa Clara, CA). All the microarray experiments were completed in triplicate.

Data analysis

MiRNA microarray images were analyzed using GENEPIX PRO. Average values of the replicate spots of each miRNA probe were background subtracted; log2 transformed and normalized using the global median using and the BRB array tools (http://linus.nci.nih.gov/BRB-ArrayTools.html). A filtering step was performed to remove probe sets that did not show significant variation across the samples: a probe was excluded if less than 20 % of expression data have at least a 1.5 -fold change in either direction from gene's median value or the percent of data missing or filtered out exceeds 50 %. For the Affymetrix experiments, Cel. Files generated by the GeneChip scanner were imported to the BRB software tools. GCRMA procedure was used for background subtraction and normalization. A filtering step was performed as described for miRNAs. To identify mRNAs- miRNAs correlations, we performed quantitative trait analysis (Spearman correlation test) using the BRB tools. To asses whether certain biological terms were enriched or over-represented in a signature, we used the **D**atabase for **A**nnotation, **V**isualization and 7 **I**ntegrated **D**iscovery (**DAVID**) http://david.abcc.ncifcrf.gov/.[23] An overrepresented term is one that has more associated genes (also referred to as members) in the gene-expression signature than is expected by chance. DAVID provides a rapid means to reduce large lists of genes into functionally related groups of genes to help unravel the biological content captured by high throughput technologies. DAVID resources provide not only the typical gene-term enrichment analysis (Gene-Ontology), but also new tools and functions that allow users to condense large gene lists into gene functional groups. In our analysis, we used high classification stringency and considered only terms that have $P<0.01$, after permutation corrections (Benjamini). We excluded from the analysis *miR-29a* and *-29b,* which are associated with CN-AML wt-NPM1, *MLL* translocation and chromosome 7 monosomy, because *miR-29*/mRNA correlations has been reported elsewhere by our group.[24]All the analyses were performed using BRB-ArrayTools version 3.6.0 (R. Simon and A.P. Lam, National Cancer Institute, Bethesda, MD) and using the R version 2.3.1 (R Foundation for Statistical Computing, Vienna, Austria) (Fig. 1 show an overflow of the experimental strategy). Network analysis was performed using Osprey.[25]

Target prediction analysis

Mainly two web databases of miRNA target prediction were used in this study: TargetScan (release 5.1)[26] and PicTar.[27]

In vitro transfection with synthetic miRNAs

The synthetic *miR-145, miR-128a, miR-26a* and scrambled oligonucleotide (control), were purchased from Ambion (Austin, TX). Four millions of K562 cells and KG-1a (ATCC, Manassas, VA) were nucleoporated using AMAXA (Solution V, Program T016) (Gaithersburg, MD) with 5 ug of precursor oligonucleotide and 0.5 ug GFP plasmid in a total volume of 10 ml. These cell lines were used because they exhibit low levels of these miRNAs (Data not shown).

Real-time quantification of miRNAs

The single tube TaqMan miRNA assays were used to detect and quantify mature miRNAs as previously described using PCR 9700 Thermocycler ABI Prism 7900HT and the sequence detection system (Applied Biosystems, Foster City, CA).[28] Expression normalization was performed with U44. Comparative real-time PCR was performed in triplicate, including no-template controls. Relative expression was expressed as 2ΔCt.

Apoptosis experiments

To demonstrate that selected miRNAs induces apoptosis, we transfected or infected miRNA mimics or scrambled oligonucleotides into K562 and KG-1a cells and measured Annexin V/ Propidium iodide (PI) stain (BD Pharmingen, San Diego, CA) at 24 hours after transfection using Flow cytometry.

Western blotting

Total protein extracts from K562 and KG-1a cells transfected with synthetic miRNA mimics and scrambled oligonucleotides were extracted using RIPA buffer (SIGMA, St Louis, MO). Protein expression was analyzed by Western blotting using Bim, Foxo3a and Pcaf antibodies (Santa Cruz, Santa Cruz, CA). Loading control was performed using β-Actin and Vinculin.

Results

Correlations of miRNA and mRNA expression profiles

Our first task was to generate a genomic wide database of miRNA and mRNA expression using samples from newly diagnosed AML patients. Patient characteristics are shown in Table 1. Total RNA was obtained from bone marrow or peripheral blood leukemic blasts and used for gene and miRNA expression profiling. Altogether, 17,531 gene probe sets and 402 miRNA probes were available for further analysis. The miRNA and mRNA expression profiles were then integrated to identify functional relationships that may contribute to AML. In order to achieve this goal, we performed correlations of expression profiles between miRNAs and mRNAs. Instead of performing correlation with all miRNAs, we focus on miRNAs whose expression levels changed the most across the AML subtypes. Thus, we selected miRNAs that are part of signatures associated with cytogenetics, molecular subtypes and outcome in AML.[9–16] Since, most of the patients in our cohort have CN-AML, we selected miRNAs that are associated with the different molecular subgroups of CN-AML, such as: *miR-10a, miR-10b, miR-145, miR-146a, miR-196a, miR-204, miR-23a, miR-26, miR-128a, miR-125a, miR-130a, let-7a, miR-15a, miR-16, miR-17, miR-20a, miR-106a* and *miR-25* (*NPM1* mutated signature)[10,13]; *miR-181a, miR-181b* (CEBPA signature and outcome[11–12]); *miR-155* (FLT3-ITD signature[9–10,13–14]). In addition we selected *miR-126, miR-223* which has been deregulated in core binding factor leukemias (t(8;21) and inv16) and in CN-AML with *MN1* mutations[13–14,16] and *miR-191, miR-326, miR-21 and miR-199a* which are associated with *MLL* translocations and outcome.[9] We next asses the relationship in the expression for each selected miRNA with the expression of the 17,521 gene probe sets in the 48 primary AML samples by using Spearman correlation

analysis within the BRB tools. In table 2, we report the numbers of probes that correlate positively and negatively with the selected miRNAs at the level of significance of $P<0.01$ (Spearman) and a false discovery rate (FDR) value <0.01. The most frequently involved miRNA was *miR-17-5p*, which correlated with 4694 gene probe sets (about 26% of all gene probes). Since some of the Affymetrix probes detect miRNA expression (i.e. *miR-155* probe) we used them as quality controls. The *miR-155* Affymetrix probe expression value was indeed positively correlated with the *miR-155* expression value detected by miRNA microarrays ((correlation coefficient) cc=0.64, $P<0.001$).

Identification of functional pathways associated with miRNA expression

To discover functional relationships between miRNAs and the transcriptome and uncover the gene pathways that are regulated by miRNAs in AML we performed gene annotation enrichment analysis using DAVID.[23] This method provides a rapid means that categorizes large lists of genes into functionally related groups of genes thereby helping in unraveling the biological content captured by genome-wide transcriptome profiling. Table 3 and 4 lists the most significant biological processes, according to David, associated with the miRNA associated gene expression signatures. We found a significant over-representation of HOX genes, in particular *HOXB* and *A* clusters, among the genes that correlated positively with *miR-20a* and the HOX embedded miRNAs; *miR-10a* and *-10b* (Table 3). Furthermore, we observed a negative correlation between *miR-181a* and *-181b and miR-155* expression with genes involved in immunity and *miR-146a, -221, -25* and *-199a* with genes involved in inflammatory response (Table 4). *MiR-23a, miR-145, miR-196a, miR-128a* and *miR-26a* expression were positively correlated with pro-apoptotic genes such as *PTEN, BIM, PDCD* and several caspases (Table 3). *Mir-23a* and *-196a* were also positively correlated with genes involved in myeloid differentiation while *miR-191, miR-222* and *miR-17* were negatively correlated with genes involved in erythroid differentiation (Table 3 and 4). The following miRNAs; *miR-17, miR-191, miR-222* and *miR-181b* were positively correlated with chromatin modifiers genes such as histone demethylating genes (Jumonji 1a), histone acetylators (*p300/CREB/GCN5*) or histone deacetylators (Sirtuin or *HDAC4*) (Table 3). *MiR-196a, miR-23a, miR-145* and *miR-126* were negatively correlated with genes involved in epigenetics (Table 4). Altogether, the data indicate an enrichment in homeobox, immunity and inflammation, apoptosis and chromatin modification biologic process among the mRNAs significantly correlated to distinctive miRNAs in AML.

Validation of the functional pathways analysis

To further test whether the functional pathways associated to specific miRNAs could be reproduced experimentally "*in vitro*", we carried out validation experiments using miRNAs involved in the apoptosis pathway i.e. *miR-145*, *miR-128a* and *miR-26a*. Furthermore, no functional data of these miRNAs have been reported in AML. Based on the miRNA-mRNA profiling integration analysis, *miR-145, miR-128a and miR-26a* expression were positively associated with pro-apoptotic genes such as *BIM*[29] and Forkhead box O3 (*FOXO3A*)[30] (Table 3), suggesting that these miRNAs could induce apoptosis in AML. To confirm this hypothesis, we ectopically over-expressed synthetic *miR-145, miR-128a and miR-26a* oligonucleotides (ODs) or scrambled ODs into K562 and KG-1a human myeloid cell lines (Fig. 2) and examined apoptosis by measuring Annexin V/propidium iodine expression using flow cytometry at 24 hours. We observed that *miR-145, miR-128a* and *miR-26a* enhanced apoptosis by 1.9, 1.35 and 2 fold, respectively in the K562 cell line and by 1.4, 1.6 and 1.3 fold, respectively with respect to the controls (scrambled ODs) in KG-1a cells (Fig. 3A and B). Next, we asked whether the apoptotic effects observed after over-expression of the *miR-145, miR-26a* and *miR-128a* in leukemic cell lines are associated with up-regulation of pro-apoptotic protein expression levels, in particular to those whose gene expression was positively correlated to the selected miRNAs. We then selected the pro-

apoptotic protein Bim (because of its critical role in apoptosis regulation)[29] and its expression was positively correlated with *miR-145* (cc=0.46, *P*=0.0009) and the pro-apoptotic Foxo3a[30], whose gene expression positively correlated with *miR-145* (cc=0.49, P=0.0004), *miR-26a* (cc=0.65, *P*=9e-07) and *miR-128a* (cc=0.5, *P*=0.0002) in 48 newly diagnosed primary AML patients. Over-expression of *miR-145* resulted in a higher Bim protein expression in K562 and KG-1a cells compared with the control (Fig. 3C), while no changes were observed for *miR-26a* and *miR-128a* at 72 hours (both miRNAs did not have any correlation with Bim in primary AML samples) (Data not shown). Over-expression of *miR-128a* and *miR-26a* resulted in up-regulation of Foxo3a in K562 cells, while no changes were observed for *miR-145* and *miR-26a* in KG-1a cells (Fig. 3D). Last we investigated whether we could validate miRNA-mRNA interactions within the chromatin modification pathway. We selected the top negatively correlated gene with *miR-145*, the histone acetylator P300/CBP-associated factor (*PCAF*)[31] (cc=−0.71, *P*=1e-07) and performed immunoblotting analysis after *miR-145* or scrambled oligonucleotides transfection in K562 and KG-1a cells. Over-expression of *miR-145* induced down-regulation of Pcaf protein expression at 72 hours after transfection with respect to the control in both cell lines (Fig. 3E).

Network analysis

To determine whether different miRNAs within a signature interact with the same target genes, we performed network analysis using Osprey, which is software platform for visualization of complex interaction networks.[25] First, we examined apoptosis and focus on the miRNAs, whose expression was correlated positively with this term; *miR-145*, *miR-128a*, *miR-23a*, *miR-26a* and *miR-196a*. As shown in Figure 4A, network analysis identified that most of the transcripts are associated with more than one miRNA, suggesting a combinatorial effect in gene regulation by co-expressed endogenous miRNAs in AML. We also performed network analysis using miRNAs negatively correlated with chromatin modification genes such as; *miR-145, miR-126, miR-196a* and *miR-23a*. Similar to the apoptosis network analysis, miRNAs associated with chromatin modification pathways were correlated with the same cluster of transcripts (Fig. 4B). Last we perform network analysis using immune system/inflammation enriched miRNA-mRNA interaction pairs and identified transcripts, like leukotriene B4, NFAT activating molecule 1 and several Toll like receptor (TLR) genes, that were associated with more than one miRNA such as *miR-221, miR-181b, miR-146a, miR-155* and *miR-199a* (Fig. 4C). Altogether, our results suggest that a small group of miRNAs coordinately regulates a pathway transcriptome by modulating the same group of genes within the pathway.

Discussion

Our study indicate that by correlating data from two different platforms that allow assessment of genome-wide gene and miRNA expression profiles, putative functional miRNA-mRNA interactions could be identified in AML samples. These interactions appear to take place within pathways controlling hematopoiesis, innate immunity, apoptosis and chromatin remodeling. Many of these interactions have been experimentally validated in other tumors or disease models. For example, both *miR-221* and *miR-222*, have been shown to negatively regulate erythroid differentiation by targeting the oncogene c-*KIT*.[32] Consistent with the literature, we found that *miR-222* is inversely correlated with genes involved in erythroid differentiation. The strong association of the *HOX* embedded *miR-10a* and *miR-10b* with *HOX* genes was also previously reported [9–10].

Concerning miRNAs correlated positively with apoptosis in our study (Table 3), *miR-145* has been described to exhibit pro-apoptotic effects in breast and bladder cancer cells.[33–34] The pro-apoptotic effects of *miR-145* in breast cancer are dependent on *TP53* activation, and

TP53 activation can, in turn, stimulate *miR-145* expression, thus establishing a death-promoting loop between *miR-145* and *TP53*.[33] We further validated this findings experimentally "*in vitro*" by transiently over-expressing *miR-145* in AML cell lines. We found that *miR-145* induced apoptosis and increased the expression of the pro-apoptotic protein Bim[29], whose gene correlated positively in primary AML samples. Though, *BIM* is regulated by *miR-145*, it does so indirectly since there are no predicted sites for direct miRNA-mRNA interaction.[26–27]

Two other miRNAs involved in the pro-apoptotic signature, *miR-23a* and *miR-26a,* when over-expressed, they induced apoptosis in human embryonic kidney cells and liver cancer cells, respectively.[35–36] Furthermore, *miR-26a* has been shown to exhibits potent anticancer effects in a *MYC*-driven liver cancer mouse model.[36] We also validated "*in vitro*" the pro-apoptotic effects of *miR-23a, miR-26a* and *miR-128a* in two myeloid leukemic cell lines and measured the pro-apoptotic protein Foxo3a, whose mRNA was positively correlated with the above miRNAs in primary AML samples. While we observed increase of Foxo3a after *miR-128a* and *miR-26a* transfection in K562 cells, no changes were observed for *miR-145*. We also observed no Foxo3a expression changes after *miR-145* or *miR-26a* in KG-1a, while *miR-128a* resulted in a minor increase in the Foxo3a protein level. This result underscores the limitations of using cell lines for validation of primary AML samples. It is probable that many miRNA-mRNA interactions occurs only in the context of certain cytogenetics and molecular subtypes of AML and could not be reproduced by artificially manipulating miRNA expression in selected cell lines.

The link between *miR-155*, *miR-181* and *miR-146* with immunity and inflammation is well documented, including in AML.[12,37–40] Two knockout mouse models have demonstrated the critical role of *miR-155* in immunity: *BIC/miR-155*−/− mice have defective dendritic cell functions, impaired cytokine secretion, and Th cells intrinsically biased toward Th2 differentiation.[37–38] Over-expression of *miR-181* family members has been found up-regulated in CN-AML with *CEBPA* mutations and associated with good outcome in high risk CN-AML.[11–12] Furthermore, using mRNA gene-chip microarray analysis, the authors showed an inverse correlation between the expression of *miR-181* and genes that encode for proteins involved in pathways of innate immunity mediated by Toll-like receptors (*TLR2, TLR4, TLR8*) and interleukin-1b (NOD-like receptors *CARD8*, *12, 15* and *CASP1*).[12] Our results identified all three miRNAs associated with genes involved in innate immunity and inflammation such as *TLR4, TLR8, IRF8* and *IL6R* genes among others.

Interestingly, network analysis revealed that miRNAs that correlated with genes within a functional category, such as apoptosis, seems to regulate the same genes (e.g. phosphatase and tensin homolog; *PTEN or FOX03A*), suggesting that these are key transcripts under extensive miRNA control. It is also possible that not all correlated miRNAs are regulating simultaneously these transcripts in each AML patient and some play a larger role than others depending on the AML cytogenetic and molecular signatures.

Our study provides with many functional miRNA: mRNA relationships that will need to be explored mechanistically for their involvement in myeloid leukemogenesis. Gain and loss of function studies will be needed to investigate the role of miRNAs correlated with chromatin modification, apoptosis and immune function genes. Finally, the discovery of previously unidentified functional relationships may lead to the development of novel therapeutic approaches. Targeting miRNAs that control the aberrant pathways identified here such as innate immunity, apoptosis and chromatin remodeling, by either drugs or miRNA-based approaches may improve treatment response in AML.

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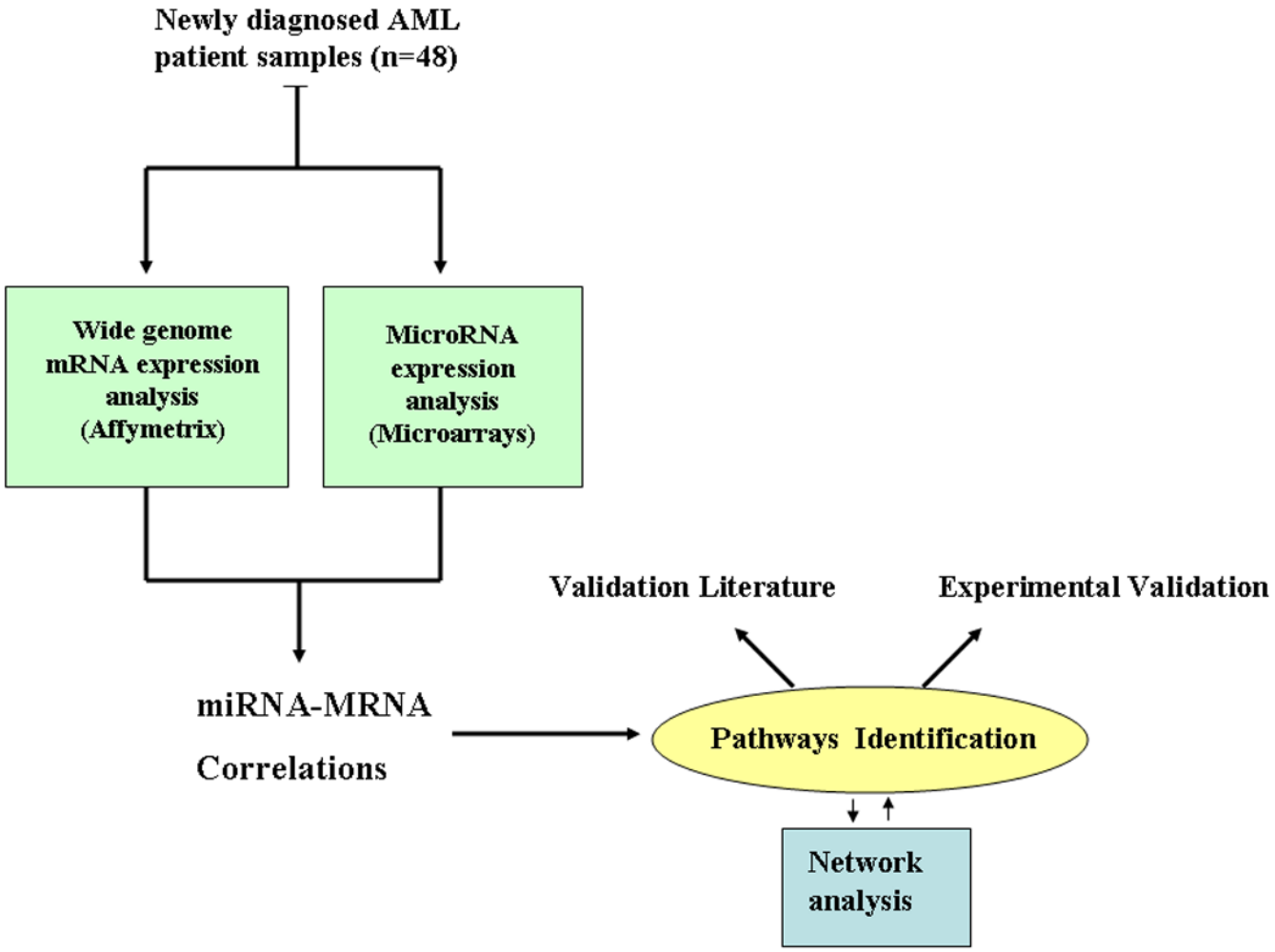

Figure 1. Study Schema and workflow

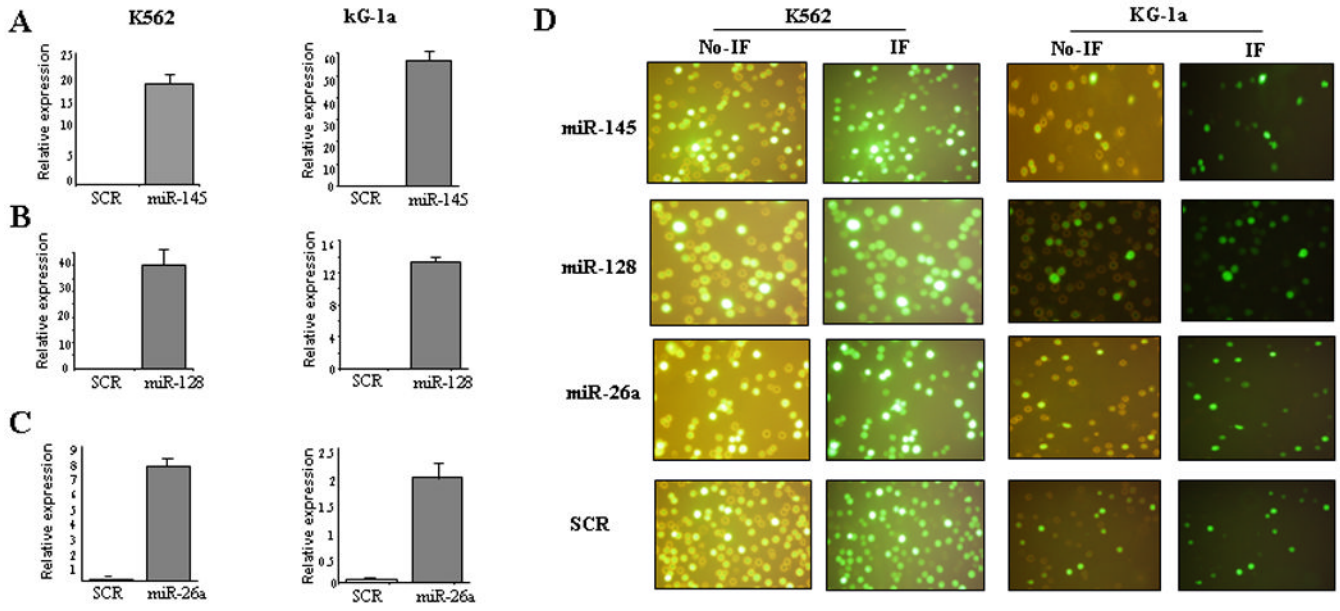

Figure 2. Expression levels of selected miRNAs and transfection efficiency after ectopic synthetic miRNA oligonucleotide nucleoporation in AML cell lines

Relative expression of *miR-145, miR-128a, miR-26a* (**A, B, and C**) in K562 and KG-1a cells after over-expression of synthetic miRNAs (*miR-145, miR-128a* and *miR-26a*) or scrambled oligonucleotides (SCR). The results are shown as relative expression after 2ΔCt calculations. **(D)** Representative images of K562 and KG-1a cells co-transfected with scrambled-GFP and selected miRNAs-GFP. The images were obtained using a Nikon Eclipse 800 microscope with filter sets for collection of fluorescent images from GFP. Images were captured with a film-based camera. Processing/analysis of images were performed using the MetaMorph software package (Universal Imaging, Inc.). The images in the left panel are from direct microscopy and the images from the right are obtained using the FITC filter (Immunofluoresence(IF)).

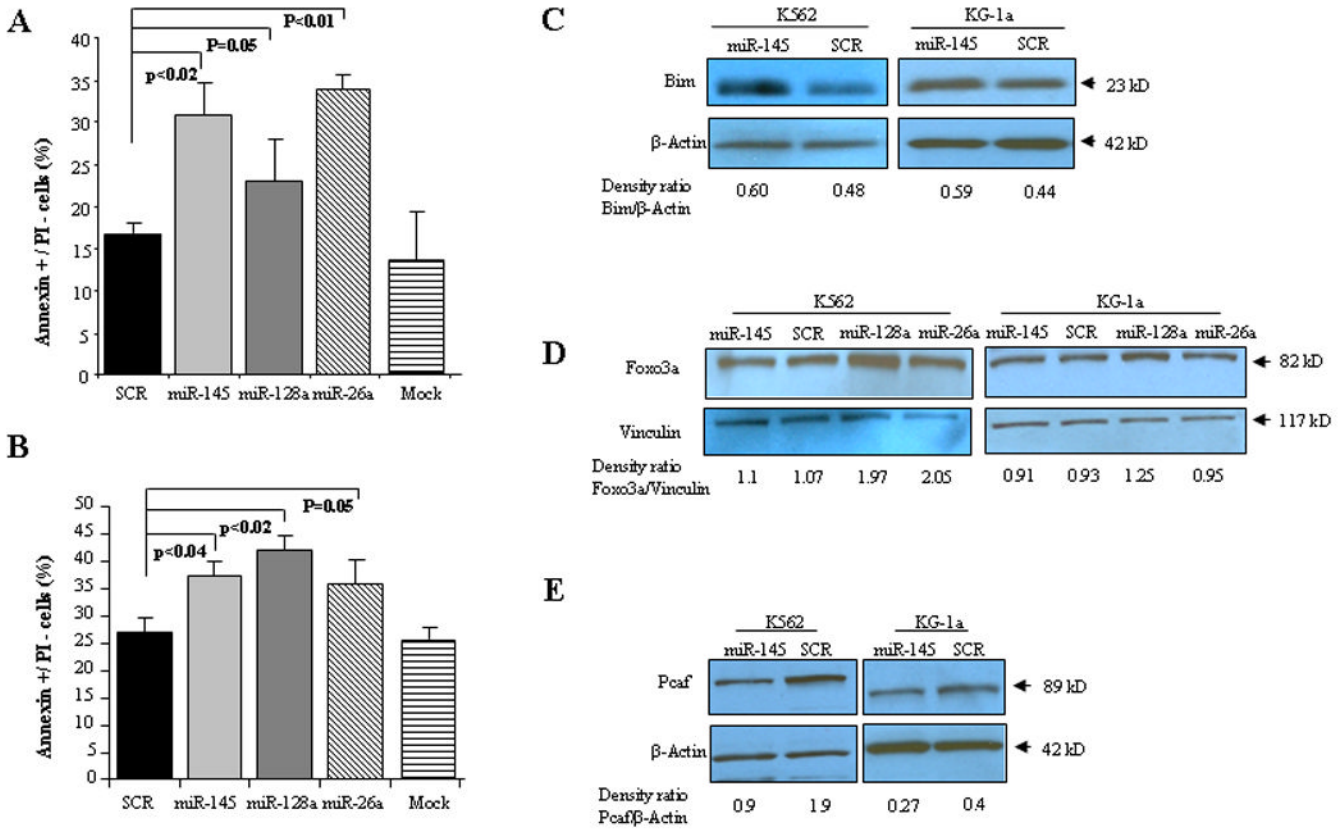

Figure 3. Experimental validation of miRNAs associated with apoptosis pathway

A) Annexin V/PI staining in K562 cells after 24 hours of transfection with water (mock), scrambled oligonucleotides (SCR), *miR-145, miR-128a* or *miR-26a*. Bar represents SD± from three independent experiments. **B)** Annexin V/PI staining in KG-1a cells after 24 hours of transfection with water (mock), scrambled oligonucleotides (SCR), *miR-145, miR-128a* or *miR-26a*. Bar represents SD± from three independent experiments. P-values were obtained by using t-test. **C)** Immunoblotting for Bim in K562 and KG-1a cells after 72 hours of transfection with *miR-145* or scrambled oligonucleotides (SCR). **D)** Immunoblotting for Foxo3a in K562 and KG-1a cells after 72 hours of transfection with *miR-145, miR-128a, miR-26a* or scrambled oligonucleotides (SCR). **E)** Immunoblotting for Pcaf in K562 and KG-1a cells after 72 hours of transfection with *miR-145* or scrambled oligonucleotides (SCR). Loading control was performed with β-Actin. Densitometry for each band was performed as per methods section and results are shown as a density ratio between target protein/β-Actin.

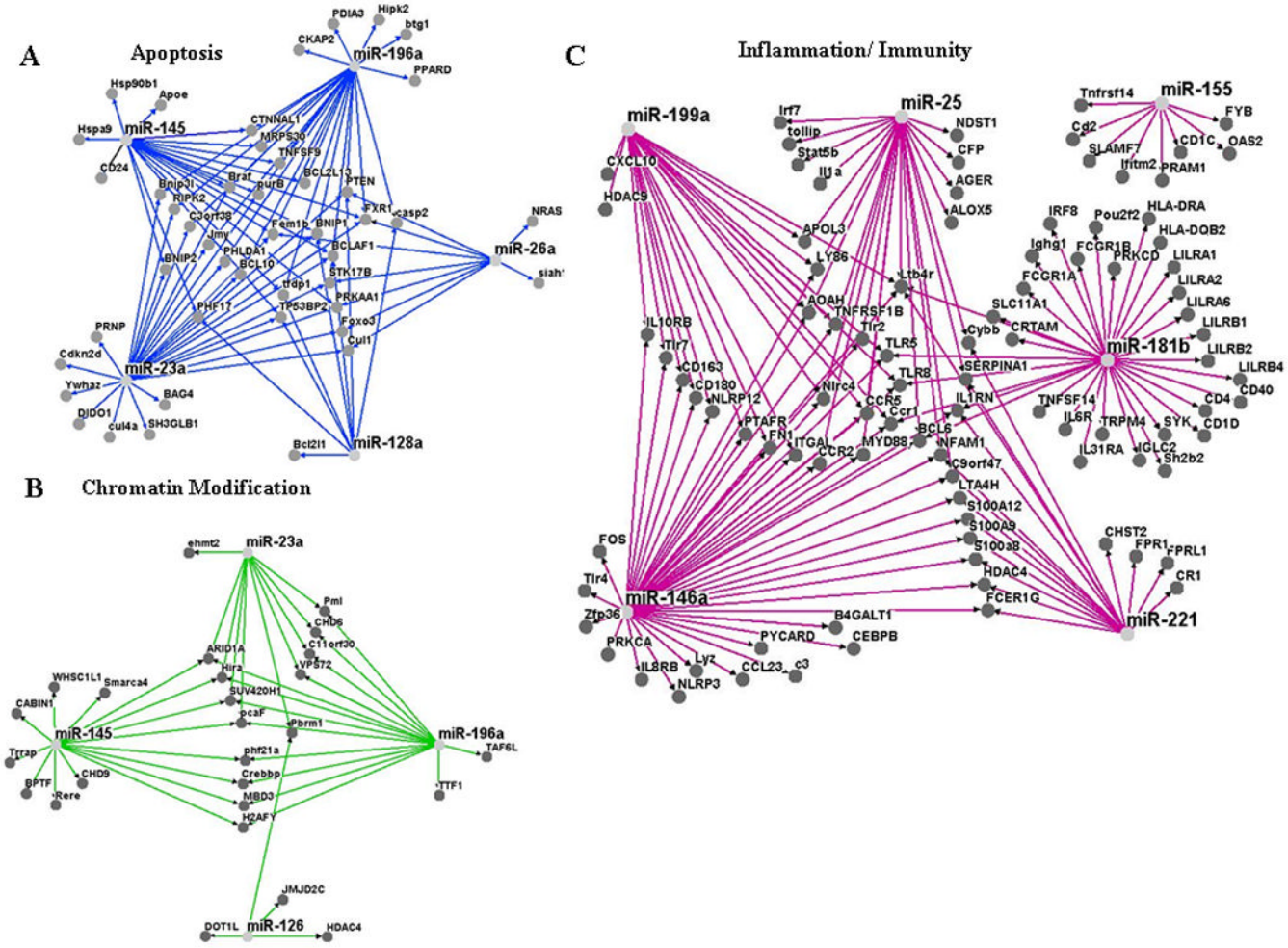

Figure 4. Network Analysis in AML

A) Apoptosis network analysis using miRNAs that positively correlated with pro-apoptosis transcripts. **B)** Chromatin Modification network analysis using miRNAs that negatively correlated with chromatin modification related genes. **C)** Inflammation/Immunity network analysis using miRNAs that negatively correlated with inflammatory/immune related genes. All the network analysis were performed using Osprey.[25]

Table 1

Patient Characteristics.

	Value
Age	
Median	59.1
Range	23–81
Sex. No. %	
Female	16 (33)
Male	32 (67)
Bone marrow blasts (%)	
Median	61.3
Range	25–94
FAB-No. %	
M0–M1	8(16)
M2	7(14)
M3	4(9)
M4–M5	26(54)
Unknown	3(7)
Cytogenetics-No %	
Normal Karyotype	32(69)
FLT3-ITD	12
FLT3-TDK	7
NPM1	20
inv16	2 (4)
t(11q23)	8 (13)
t(8;21)	1 (2)
t(15;17)	4 (9)
del9q	1 (2)

Table 2

Correlation between miRNAs and mRNAs in AML.

	Number of genes		
miRNA	**Positive correlation**	**Negative correlation**	**Total number of genes**
let-7a	220	220	440
miR-10a	700	375	1075
miR-10b	1097	871	1968
miR-15a	95	148	243
miR-16-1	721	491	1212
miR-17-5p	3094	1600	4694
miR-20a	145	182	327
miR-21	85	347	432
miR-23a	1326	2348	3674
miR-25	783	1307	2090
miR-26a	987	1311	2298
miR-30a	520	65	585
miR-106a	79	195	274
miR-126	1117	1694	2811
miR-128a	857	1776	2633
miR-130a	709	967	1676
miR-145	1075	2738	3813
miR-146	1129	1391	2520
miR-155	252	182	434
miR-181a	1195	522	1717
miR-181b	1469	1201	2670
miR-191	608	461	1069
miR-196a	1394	2959	4353
miR-199a	668	1668	2336
miR-204	107	107	214
miR-221	452	446	898
miR-222	2736	1514	4250
miR-223	269	166	435
miR-326	110	130	240

Table 3

Functional pathways analysis of positively correlated genes.

	David pathways (GO, Interpro)				
miRNA	**Positive correlation**	**P-value**	**Benjamini**	**Gene N0***	**Gene ID**
Let-7a	**RNA binding**	8.70E-06	9.20E-03	20	PRKA, HNRPM, RBM26, EIF3B, DDX24, SPEN
miR-10a	**Homeobox**	6.90E-09	2.00E-05	23	HOXB2,HOXB3,HOXB4,HOXB5, HOXB6, PBX3
miR-10b	**Homeobox**	2.30E-12	1.40E-09	25	HOXB2,HOXB3,HOXB4,HOXB5, HOXB6, PBX3
miR-15a	**None**				
miR-16-1	**None**				
miR-17-5p	**Chromatin modification**	7.10E-13	2.70E-10	54	PCAF, WHSC1, HDAC9, JHMDH2, NCOR1
miR-20a	**Homeobox**	8.00E-16	4.60E-12	10	HOXB4, HOXB5, HOXB6, HOXA3,HOXA4-A7
miR-21	**None**				
miR-23a	**Apoptosis**	3.10E-14	1.80E-11	91	PTEN, BIM, DIDO1, FOX03, PPP1R15a, BCL2L13
	Myeloid cell differentiation	1.20E-04	7.50E-03	9	CEBPB, CSF1, CEBPG, FOX03, SP3, JAG1
miR-25	**none**				
miR-26a	**Apoptosis**	1.90E-07	3.20E-05	60	PTEN, PP2A, DIDO1, HSPA1A, PDCD4, PAWR
miR-30a	**mRNA processing**	3.80E-08	6.90E-05	20	CDC5, FIP1L1, WTAP, PAPOLA, DXH35, HNRPH1
	RNA Binding	4.50E-11	3.90E-09	41	FXR1, PDCD4, RBM22, DIS3, DHX9, EIF2AK2
miR-106a	**None**				
miR-126	**none**				
miR-128a	**Apoptosis**	2.90E-08	7.90E-06	57	PTEN, CD28, FOX03A, DIDO1, TP53BP2, PHF17
miR-130a	**Zinc finger**	3.80E-08	2.80E-05	46	SP4,WT1, ZEB1, FOXP1, ZNF395, ZNF273-4
miR-145	**Apoptosis**	2.90E-14	1.50E-10	81	PRKAA1, PTEN, FOX03A, BIM, DIDO1, BCL2L13
miR-146	**Regulation of transcription**	1.80E-12	9.60E-10	129	Aebp1, E2f5, RYBP, WT1, ETV6, RUNX1
miR-155	**none**				
miR-181a	**Regulation of transcription**	1.70E-14	4.50E-11	155	GATA2, SLA2, MLL, PIA2, FOXP1, CHD9
miR-181b	**Chromatin modification**	4.50E-05	1.10E-02	15	SET, JHMDH1,ASH1, RCC1, MYST4, TLK1
miR-191	**Chromatin modification**	2.00E-09	1.30E-06	23	MLL3, MYST4, NCOR1, HDAC4, HDAC9, EHMT2
miR-196a	**Apoptosis**	6.20E-13	4.10E-10	90	PTEN, BAG4, PPARD, TP63, FXR1, CKAP2
miR-199a	**Regulation of transcription**	5.40E-06	3.20E-03	80	GATA3, MAX, RORA, FOXP1, ERG, AML1
miR-204	**None**				

David pathways (GO, Interpro)

miRNA	Positive correlation	P-value	Benjamini	Gene N0*	Gene ID
miR-221	**None**				
miR-222	**Chromatin modification**	2.70E-08	1.40E-05	27	MCBD3, EHMT2, SET, HELLS, H2AFY, SMYD3
miR-223	**RNA binding**	1.20E-07	6.50E-05	26	UMHK1, HINT3, UNC13D, SFRS6, SFRS11, GRAP
miR-326	**None**				

* number of genes enriched for that pathway

Table 4

Functional pathways analysis of negatively correlated genes.

	David pathways (GO, Interpro)				
miRNA	**Negative correlation**	**P-value**	**Benjamini**	**Gene N0**	**Gene ID**
Let-7a	**None**				
miR-10a	**none**				
miR-10b	**Gtpase activator activity**	4.10E-07	1.20E-03	28	AKAP13, TBC1D10, TRIO, PSD3, NUP62
miR-15a	**Zinc finger region C2H2**	1.50E-08	6.40E-06	12	ZFP225, FFP227, ZFP45, ZPF600, ZFP85
miR-16-1	**none**				
miR-17-5p	**Hematopoiesis**	4.20E-05	3.50E-03	27	TAL1,SP3, CEBPB,CSF1, AHSP, FOX03A
	Apoptosis	3.80E-15	2.00E-11	103	BIM, PTEN,CXCR4, PLAGL2, DEDD,STK38
miR-20a	**None**				
miR-21	**Krueppel-box (Zinc Finger)**	4.90E-15	1.90E-12	29	ZFP141, ZFP192, ZFP304, ZFP253, ZFP606
	Cell cycle	2.70E-11	1.50E-08	41	E2F8, CDC25, CDCA2, CDCA8, NPM1,RIF1
	Mitosis	3.60E-12	2.00E-10	20	KIF11, CENPF, CENPE, HELLS, EB1, KNTC1
miR-23a	**Chromatin modification**	1.60E-08	2.70E-06	39	NCOR1,CHD9, JARID1A, HDAC4, TTF, DOT1L
miR-25	**Immune response**	1.00E-09	5.30E-07	77	Ltb4r, NFAM1, BCL6, MYD88, CCR2, LY86
	Hematopoiesis	6.70E-05	1.10E-02	21	STAT5b, KLF6, JAK2, SP1, ITPKB, DLL4
miR-26a	**None**				
miR-30a	**None**				
miR-106a	**None**				
miR-126	**Chromatin modification**	5.10E-06	1.10E-03	29	CHD9, SUV42C, CHD6, Men1, CBX7, CHD3
miR-128a	**Gtpase activator activity**	1.90E-07	7.70E-05	38	ARF6, SH2B2, CRK, TBC1D14, ARHGAP27
miR-130a	**Lysozome**	3.89E-09	1.40E-06	28	CD1B, GM2a, CTSL1, CTSL1, IGF2R, CXCR2
miR-145	**Chromatin modification**	1.60E-07	2.90E-05	46	SIRT2, JMID2b, TAF6L,BPTF, MEN1, HIRA
miR-146	**Inflammatory response**	1.40E-10	1.20E-07	42	TLR4, CCl23, Ltb4r, LYZ, IL8RB, MYD88
miR-155	**Immune response**	3.10E-06	1.60E-02	23	SHIP-1, CD2, FYB, PRAM1, CD1C, IRF7
miR-181a	**Immune response**	7.50E-07	4.00E-03	31	IL1RN, BCL-6, CCR1, PTAFR, FN1, CCL2
miR-181b	**Immune response**	2.80E-06	3.00E-03	35	TLR5, TLR8, LTb4r, IL6R, TNFSF1
miR-191	**Erythrocyte differentiation**	2.20E-12	2.30E-09	10	KLF1, ETS1, TAL1, EPB42, HBA1, HBB
miR-196a	**Chromatin modification**	8.90E-10	1.90E-07	46	JARID1B, ASH1L, BRD8, RSF1, TAF6L
miR-199a	**Inflammatory response**	1.20E-06	7.60E-04	28	CCR5, CCR9, CARD12, TLR5, TLR8,PTAFR
miR-204	**None**				
miR-221	**Inflammatory response**	3.60E-11	1.90E-07	26	NFAM1, Ltb4r, CR1, LTA4H, TLR4, BCL6
miR-222	**Erythrocyte differentiation**	1.70E-06	8.70E-03	9	TAL1, EPB42, ETS1, HBB, HBA1, HBD
miR-223	**None**				
miR-326	**Mithocondrium**	7.50E-08	1.00E-05	22	CYCS, MRPL1, MRPL19, NDFIP2