Abstract. Dysregulation of microRNAs (miRs) has been linked to several types of cancer. In the present study, we investigated the expression of miR-181a in different leukemia cell lines and healthy hematopoietic cells, as well as its influence on cell proliferation, metabolic activity and potential targets. Expression of miR-181a differed between various leukemia cell lines and mature blood cells. Inhibition of miR 181a expression in T- and B-Acute Lymphoblastic Leukemia (ALL) cells revealed an influence on the potential targets High-Mobility-Group-Protein B1 (HMGB1) and Cluster of Differentiation 4 (CD4). Overexpression of miR-181a in AML cells led to a significant decrease in cell proliferation and metabolic activity. The present data indicate a possible role of this specific miRNA in immunogenicity.

MicroRNAs (miRNAs) are a class of small, endogenous RNA molecules influencing protein expression via RNA interference. They are involved in almost every cellular process, including proliferation, apoptosis and differentiation. Expression analysis of miRNA genes revealed several dysregulated miRNAs in solid tumors, as well as hematological neoplasias (1). Thus, it is possible to discriminate acute leukemia into Acute Myeloid Leukemia (AML) or Acute Lymphoblastic Leukemia (ALL) on the basis of miRNA expression profiles (2). Several mechanisms of miRNA action have been described, mediated by base pairing between miRNA and 3'Untranslated Region (UTR). In most cases miRNA mRNA binding leads to reduced mRNA expression, caused by increased degradation or inhibited translation (3, 4). However, three recently published studies have shown an activating effect of miRNA binding on translation (5-7).

miR-181a is part of a conserved family consisting of four members (miR181a to d). Detailed analysis of human blood cells revealed a high expression of miR-181a in hematopoietic stem cells (HSCs), whereas expression in mature hematopoietic cells was considerably lower (8). Two studies investigated the role of miR-181a during T-cell development and activation (9, 10). Li et al. identified the activation markers Cluster of Differentiation 5 (CD5) and CD69 as direct targets of miR-181a and described an inhibiting role of miR-181a in T-cell activation (10). In addition, the oncogene K-RAS (11) and the transcription factor Prospero homeobox protein 1 (Prox1) (12) were identified as direct targets of miR-181a. A study of Debernardi et al. correlated the miR-181a expression with a specific morphological subtype in de novo AML samples with normal karyotype (13). A high miR-181a expression was associated with an immature subtype. Additionally, in silico approaches based on mRNA expression data identified a number of potential miR-181a targets. One of them was HMGB1, a member of the high mobility group box DNA-binding proteins. It has two DNA-binding motifs, which bind non-specifically to the small groove of the DNA to bend it, so that other factors can bind and activate transcription. Additionally, HMGB1 has a pro-inflammatory cytokine domain. The expression of HMGB1 was positively correlated with miR-181a expression (13). Moreover, HMGB1 mRNA possesses a potential miR-181a binding site (14). The CD4 co-receptor is another potential target of miR-181a as the 3'UTR of CD4 mRNA possesses a miR-181a binding site at nucleotides 74-81 (15). CD4 plays a major role in activation of T-helper cells. Both potential targets (HMGB1 and CD4) might be of importance in acute leukemia since they possibly influence immunoreactivity if their expression is altered. Here, we analyzed the expression and functions of miR-181a in AML and ALL, as well as high grade Non-Hodgkin lymphoma (hgNHL) in more detail.

Materials and Methods

De novo cells and cell lines. HSCs were isolated from bone marrow of volunteers. After ficoll density gradient centrifugation, cells were enriched for CD34+ cells using an autoMACSTM separator (Miltenyi Biotec, Bergisch Gladbach, Germany). B-Cells, monocytes and
RNA isolation and quantitative real-time Polymerase chain reaction (PCR). RNA was isolated from all samples using the miRNeasy Kit from Qiagen (Hilden, Germany) according to the manufacturer’s protocol. Synthesis of cDNA and real-time PCR were performed using TaqMan MicroRNA Assays from Applied Biosystems (Darmstadt, Germany). For first-strand cDNA synthesis, 10 ng total RNA were used with 50 nM stem loop primer, 1× Reverse Transcription (RT) buffer, 50 U Multiscribe RT and 38 U RNase inhibitor in a final volume of 15 μl. The mix was incubated for 30 min at 16˚C, 30 min at 42˚C, 5 min at 85˚C. Real-time PCR was performed using a standard TaqMan® protocol. The 20 μl PCR reaction contained 1.33 μl RT product, 1× PCR MasterMix (Eurogentec, Köln, Germany), 1× TaqMan® probe/primer Mix (Applied Biosystems). The reactions were carried out in a 96-well plate at 95˚C for 10 min followed by 40 cycles of 95˚C for 15 s and 60˚C for 1 min. All PCR reactions were run in triplicate and included no template controls. The amount of RNA was normalized to that of the small nuclear RNA c/d Box 48 (RNU48).

Flow cytometry. Apoptosis and necrosis rates were determined using annexin V Fluorescein isothiocyanate (FITC; BD Biosciences, Heidelberg, Germany) and the propidium iodide (PI) (Sigma Aldrich, St. Louis, MO, USA) labeling technique and flow cytometric analyses, as described by others (17). Unstained and single-stained controls were included in each experiment. The following antibodies were used: IgG1-FITC, CD19-FITC, CD20-FITC, CD3-FITC, CD4-FITC, CD5-FITC, CD14-PE, CD33-PE, CD38-PE (all from BD Biosciences). Flow cytometry analyses were performed using a FACSCalibur instrument (BD Biosciences) and the data were analyzed with CellQuest software (BD Biosciences).

Statistical analysis. Relative quantification of miRNA expression was calculated using the Relative Expression Software Tool© (18, 19). The experiments were conducted in triplicates and results within each experiment were described using the mean±standard deviation. Significance between control and treated cells was calculated using the Student’s t-test. A p-value less than 0.05 was considered to be significant.

Results

Expression of miR-181a in leukemia cell lines and mature hematopoietic cells. Expression of miR-181a was determined in mature de novo cells, as well as in neoplastic cell lines, in relation to the expression in HSCs (Figure 1). Overall, miR-181a expression was heterogeneous and not associated with lymphoid or myeloid background. Mature peripheral blood cells (B-cells, monocytes, granulocytes) exhibited lower miR-181a expression compared to HSCs. The two T-ALL cell lines exhibited a heterogeneous expression of miR-181a. Whereas expression of miR-181a was very high in MOLT-4 cells, the expression in Jurkat cells was similar to that of HSCs. All three B-ALL cell lines had a significantly higher miR-181a expression than did HSCs with the highest expression in REH cells. The hgNHL cell line DogKit also exhibited a very high expression, while DoGum and GumBus displayed a lower expression than did HSCs. Expression of miR-181a in the AML cell lines MV4;11 and HL60 was also lower than in HSCs, in NB4 cells it was higher.

Influence of miR-181a inhibition on cell proliferation and metabolic activity. For inhibition experiments, the cell lines REH and MOLT-4 were used, as they express high levels of...
miR-181a (Figure 1). Transfection with an LNA-modified ASO led to a strong and long-lasting reduction of miR-181a expression in both cell lines (Figure 2 A). The expression in the transfected cells was significantly lower versus both controls at every time point. To evaluate the influence of miR-181a expression on cell proliferation and metabolic activity, cell count and WST-1 assay were performed following transfections (Figure 2 B and C). The inhibition of miR-181a expression in ALL cell lines had no influence on cell proliferation nor on metabolic activity. Determination of apoptosis and necrosis rates by flow cytometry showed no differences between the samples (data not shown).

Influence of miR-181a inhibition on surface marker expression. In order to detect an influence of miR-181a on the expression of different cell surface markers, flow cytometry experiments were performed. The investigated surface proteins have been known to be influenced by miR-181a [CD5, CD69 (10)] or have been described as having potential binding sites for miR-181a in their 3’UTRs (CD4, Figure 3 A). Additionally, the expression of CD34 and CD38 as differentiation markers and of CD3, CD19, CD33 and HLA-DR as activation markers were examined. There were no differences in the percentages of positive cells between the control samples and the miR-181a-inhibited samples (data not shown). Nevertheless, the mean fluorescence intensity (MFI) of CD4 increased over time in MOLT-4 cells (Figure 3 B). Calculation of relative MFI (rMFI) showed a significant increase of CD4 15 h and 24 h after miR-181a inhibition (p=0.008 and 0.003), respectively.

Influence of miR-181a inhibition on HMGB1 expression. The influence of miR-181a on the expression of HMGB1 was determined at the protein level by western blot (Figure 3 C). The inhibition of miR-181a led to a strong reduction of the HMGB1 protein in both tested ALL cell lines (REH,
MOLT-4). After 15 h and 24 h, the protein levels were reduced compared to both controls, and after 48 h and 72 h HMGB1 was no longer detected in miR-181a-inhibited samples at all.

Overexpression of miR-181a in HL60 cells. In order to discover potential effects of miR-181a overexpression, the AML cell line HL60, which has a low miR-181a expression compared to HSCs (Figure 1), was transfected with a miR-181a mimic. Transfection with the miR-181a-specific oligonucleotide led to strong overexpression of miR-181a (Figure 4 A). The miR-181a expression induced a reduction in metabolic activity and cell proliferation compared to both controls (Figures 4 B and C). After 24 h and 72 h, the metabolic activity was significantly lower in transfected cells compared to the controls. Cell proliferation decreased after 48 h. No changes in apoptosis rates were detected (Figure 4 D). The necrosis rate was significantly higher in miR-181a overexpressing cells compared to controls after 72 h (Figure 4E).

Discussion
The expression of miRNAs in different diseases has been intensively evaluated (8) and some links between dysregulated miRNAs and distinct types of cancer have been described (1). miR-181a was shown to play a role in the maintenance of stem cell state and to be dysregulated in different hematopoietic neoplasias (20-22). In the present study, we evaluated the expression of miR-181a in different leukemia and hgNHL cell lines and performed functional analyses to reveal possible roles of miR 181a in hematopoietic cells. Expression of miR-181a varied between different cell populations and no schema based on phenotype could be identified. miRNA expression might depend upon other factors, such as gene copy number (i.e. duplications or deletions of chromosomes) as well as on the differentiation stage of the cells. All three mature cell populations (B-cells, monocytes, granulocytes) exhibited lower expression of miR-181a compared to HSCs. A similar expression profile with the
The highest miR-181a expression in immature stem cells was described by Chen et al. and Landgraf et al. (8, 23). miR-181a expression differed considerably between the various cell lines. Whereas MOLT-4 displayed the highest miR-181a expression of all cell lines, miR-181a expression in Jurkat was similar to that of HSCs. The expression in the three B-ALL cell lines was uniformly higher than that in HSCs. This is consistent with published studies: the expression of miR-181a in pediatric B-ALL was evaluated by Schotte et al. in 2008 (24). They found a high miR-181a expression in de novo ALL samples compared to HSCs. The hgNHL cell line DogKit carries a duplication of chromosome 1, which harbors one gene locus of miR-181a (16). Therefore, the high expression in this cell line may be at least in part due to this abnormality. The other hgNHL cell lines DoGum and GumBus exhibited a reduced miR-181a expression compared to HSCs. This is in line with their higher maturation grade compared to HSC and ALL cell lines. In AML cells with a normal karyotype, Debernardi et al. showed there to be higher miR-181a expression in immature (FAB M1, M2) than in more mature subtypes (FAB M4, M5) (13). The AML cell lines presented here do not fit into this schema, as each of our cell lines carried different cytogenetic aberrations. The data presented here as well as other data from the literature, support a role for miR-181a during hematopoietic maturation with an increase in the early stages and a decrease in more mature stages of development (9). The transfection with LNA-modified ASOs led to a strong reduction of miR-181a expression, while transfection with miR-181a induced a strong overexpression of miR-181a. Nevertheless, only miR-181a

![Figure 3](image_url)

Figure 3. miR-181a influences Cluster of Differentiation 4 (CD4) and High-Mobility-Group-Protein B1 (HMGB1) protein expression in MOLT-4. A: miR-181a binding site in CD4 and HMGB1 3' UTR, provided by targetscan.org, version 5.1. B: Relative mean fluorescence intensity (rMFI) of CD4 in MOLT-4 cells was significantly increased 15 h and 24 h after miR-181a inhibition compared to mock-transfected cells. Results are displayed as the means±SD of three independent experiments (*p<0.05). C: HMGB1 protein expression decreased as early as 15 h after miR-181a inhibition in REH and MOLT-4 cells. A representative western blot of three independent experiments is shown.
overexpression influenced cell proliferation, viability and metabolic activity. We suppose that this may be due to the fact that an increase of miRNA expression may have a broader influence on cell functions. A huge excess of a miRNA may lead to an effect that is not seen under endogenous conditions. It will lead to more mRNA-miRNA complexes that would not be otherwise stable. One of the proteins with a possible miR-181a binding site that could be responsible for the antiproliferative effect due to miR-181a overexpression is the oncogene FBJ murine osteosarcoma viral oncogene homolog (FOS), which is regulated by the Moloney murine sarcoma viral oncogene homolog (MOS)/ Dual specificity mitogen-activated protein kinase kinase (MEK)/ Mitogen-activated protein kinase (ERK) pathway (25, 26). The 3'UTR of Endoplasmin (HSP90B) shows also a possible miR-181a-binding site (25). This protein has proproliferative effects and is cleaved during necrosis by members of the calpaine family (27). A reduction of HSP90 due to miR-181a overexpression may mimic the effect of cleavage. Several surface proteins have already been described as targets of miR-181a (10). In this study, we evaluated the expression of various differentiation and activation markers after miR-181a inhibition. Only the expression of CD4 was influenced by miR-181a inhibition. Although there were no differences in the number of CD4-positive cells, an effect was seen on the MFI, i.e. the density of CD4 on the cell surface. As miR-181a regulates the stability and translation of CD4 mRNA, but not its transcription, it is consistent to see no increase in the number of CD4-positive cells. The slight increase in the rMFI implicates the role of other miRNAs in the regulation of CD4 expression. Major candidates are the other three members of the miR-181 family (miR-181b to d), as they are highly homologous. A second reason could be due to the half-life of CD4 protein. Since miRNAs influence only the neogenesis of proteins, a long half-life leads to a weak effect of miRNA inhibition. Indeed, the rMFI of CD4 increased over the
complete observation period, even though the differences in rMFI were not significant at later time points. The expression of HMGBl was described to correlate with miR-181a expression in patients with AML (13). In the present study, we showed a decrease of HMGBl protein after miR-181a inhibition in ALL cell lines. As the majority of miRNA effects correlate negatively, this is an uncommon effect. However, other publications have shown a positive correlation between miRNA expression and the expression of its targets (5-7). Even if the 3'UTR has a potential binding site, it is still possible that miR-181a indirectly affects HMGBl expression. It remains to be shown if dysregulation of HMGBl, perhaps due to miR-181a dysregulation, promotes leukemogenesis. The regulation of CD4, as well as HMGBl by miR-181a might be of importance, especially in leukemia, as both proteins are important immune modulators. Their dysregulation might influence allogenic graft versus leukemia effects following stem cell transplantation.

In conclusion, we demonstrated a heterogeneous expression of miR-181a in leukemia and lymphoma cell lines compared to healthy HSCs and mature blood cells. Overexpression of miR-181a in AML cells led to an inhibition of cell growth and metabolic activity, while inhibition of miR-181a in ALL had no effect on these processes. The expression of CD4 and HMGBl was influenced by miR-181a. However, the importance of these regulations in acute leukemia remains to be further defined.

Conflicts of Interest

The Authors have no conflicts of interest to report.

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