PML/RARα-Regulated miR-181a/b Cluster Targets the Tumor Suppressor RASSF1A in Acute Promyelocytic Leukemia

Daniela Bräuer-Hartmann, Jens-Uwe Hartmann, Alexander Arthur Wurm, Dennis Gerloff, Christiane Katzerke, Maria Vittoria Verga Falzacappa, Pier Giuseppe Pelicci, Carsten Müller-Tidow, Daniel G. Tenen, Dietger Niederwieser, and Gerhard Behre

Abstract

In acute promyelocytic leukemia (APL), all-trans retinoic acid (ATRA) treatment induces granulocytic maturation and complete remission of leukemia. microRNAs are known to be critical players in the formation of the leukemic phenotype. In this study, we report downregulation of the miR-181a/b gene cluster in APL blasts and NB4 leukemia cells upon ATRA treatment as a key event in the drug response. We found that miR-181a/b expression was activated by the PML/RARα oncogene in cells and transgenic knock-in mice, an observation confirmed and extended by evidence of enhanced expression of miR-181a/b in APL patient specimens. RNA interference (RNAi)-mediated attenuation of miR-181a/b expression in NB4 cells was sufficient to reduce colony-forming capacity, proliferation, and survival. Mechanistic investigations revealed that miR-181a/b targets the ATRA-regulated tumor suppressor gene RASSF1A by direct binding to its 3′-untranslated region. Enforced expression of miR-181a/b or RNAi-mediated attenuation of RASSF1A inhibited ATRA-induced granulocytic differentiation via regulation of the cell-cycle regulator cyclin D1. Conversely, RASSF1A overexpression enhanced apoptosis. Finally, RASSF1A levels were reduced in PML/RARα knock-in mice and APL patient samples. Taken together, our results define miR-181a and miR-181b as oncomiRs in PML/RARα-associated APL, and they reveal RASSF1A as a pivotal element in the granulocytic differentiation program induced by ATRA in APL.

Introduction

Acute promyelocytic leukemia (APL) is characterized by specific chromosomal translocations involving the retinoic acid receptor α (RARα; refs. 1, 2). The most frequent translocation fuses the RARα with the promyelocytic leukemia protein (PML) gene (3). At physiological levels of retinoids, the PML/RARα fusion protein causes block of differentiation and neoplastic transformation by disrupting the function of PML and repression of transcription of genes regulated by RARα (2, 4, 5). Pharmacologic doses of retinoids can overcome this block, lead to the expression of granulocytic specific transcription factors like C/EBPβ (6), and thereby induce terminal differentiation of APL blasts in vitro and in vivo (1, 2).

Recent studies identified a group of small molecules that are involved in posttranscriptional regulation of gene expression. microRNAs (miRNA) are endogenous, non–protein-coding small RNAs that play critical roles in the posttranscriptional regulation of target genes by direct targeting of mRNAs for cleavage, translational repression, or destabilization (7). A selected number of miRNAs has been shown to play key roles in hematopoietic differentiation (8) as well as in the formation and maintenance of leukemia (9). We and others already showed that miR-223, miR-34a, and miR-30c are important factors in myeloid differentiation (10–13). While some miRNAs like miR-223 have been implied in APL differentiation (14) and tumorigenesis, there is still a lack of knowledge about the expression and function of other miRNAs.

In this study, we showed that the genomic clustered miR-181a and miR-181b (miR-181a/b) are highly expressed in APL and downregulated during all-trans retinoic acid (ATRA)-induced differentiation (14–16). By analyzing APL and acute myeloid leukemia (AML) patient samples as well as PML/RARα knock-in mice, we demonstrated that miR-181a and miR-181b display a very specific PML/RARα dependency in vitro. Furthermore, we revealed that miR-181a and miR-181b are involved in the formation of the PML/RARα caused oncogenic phenotype. We showed that the miR-181a/b function is determined by the direct binding to their target RASSF1A. Finally, we firstly describe the tumor suppressor RASSF1A as a new and essential member of the retinoic acid–induced differentiation network in APL.

Materials and Methods

Human cell samples from AML patients and healthy donors

AML patient samples were obtained as RNA and as frozen bone marrow samples from the University of Münster (Münster, Germany).
Germany) and from the OSHO patient sample collection (Leipzig, Germany). Blood cells samples from patients without any hematopoietic disease were obtained from the University Hospital Halle (Halle, Germany). Ethics committee approval was obtained and all patients provided informed consent. All samples were karyotyped and molecular genetic analysis was performed previously.

Transgenic mouse model

Bone marrow cells from 5 PML/RARA knockout mice (C57BI/6-mGC) in which PML/RARA is expressed under the control of the murine cathepsin G gene (C57BI/6-mGC) were karyotyped and molecular genetic analysis was performed on April 13, 2017. © 2015 American Association for Cancer Research. Published OnlineFirst June 3, 2015; DOI: 10.1158/0008-5472.CAN-14-3521

DNA constructs, cloning, and mutagenesis

For luciferase assay, RASSF1A 3’ untranslated region (3’UTR) was inserted in the pcGl3-luciferase reporter control vector downstream of the luciferase encoding region (Promega). MiR-181–binding site positions in the 3’UTR were taken from the miRNA target base datatargetscan (www.targetscan.org). The RASSF1A 3’UTR (Acc. No.[NM_007182.4]) was amplified from cDNA of NB4 cells treated with 1 μmol/L ATRA for 48 hours using primer pairs, which generate a XbaI restriction enzyme recognition site at the 5’- and 3’-ends of the amplified DNA product. The following primer pairs were used: RASSF1A 3’UTR forward, 5’-GTCTAGAGAG-GATCTGGAATTCTTGAG-3’; reverse, 5’-GTCTAGAGAG-GATCTGGAATTCTTGAG-3’. The purified DNA fragment and the pcGL3 luciferase reporter control vector were digested with XbaI and fused in a T4 ligase reaction (Invitrogen). Mutagenesis of miR-181–binding sites was done with the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer’s instructions. All sequences were verified by sequencing. pcDNA3.1/RASSF1A expression vector was a kind gift from Reinhard Dammann (Justus-Liebig University, Giessen, Germany).

Luciferase reporter assay

To prove the direct binding of miR-181a/b to the 3’UTR of RASSF1A mRNA, U937 cells were transiently cotransfected with 0.5 μg of each reporter construct (pcGL3 control vector, pcGL3/3’UTR-RASSF1A, and pcGL3/3’UTR-RASSF1A mutated), 0.1 μg of Renilla construct (pRL) and 1 μmol/L miR-181a, miR-181b mimics, or control mimics using Amaxa Technology (Lonza). Luciferase activities were determined 24 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega). Values were normalized using Renilla luciferase.

Lentivirus production and transduction

pmiR-ZIP-lentivirus vectors were purchased from System Biosciences, and p-RFP-CB-shLenti vectors were purchased from Origene. miR-ZIP-lentiviral particles were produced according to the manufacturer’s instructions. NB4 cells were infected two times within 48 hours with 10 μL of PEG–it–concentrated viral particles followed by Puromycin selection of transduced cells for additional 7 days followed by FACS-GFP sorting to create stable miR-ZIP NB4 cell lines. For generating a RASSF1A knockdown NB4 cell line, viral particles were produced by transfecting 293NT cells with a set of four different shRNA vectors and the control shRNA vector according to the manufacturer’s instructions. Selection of shRNA-expressing clones was performed by using blasticidin for 2 weeks.

Cell growth assay

NB4 cells stably transduced with pmiR-ZIP-181a, pmiR-ZIP-181b, or control vector were plated in a density of 1 × 10^5 cells/mL. Proliferation rate was ascertained by cell counting in a Neubauer chamber using Trypan blue staining for excluding dead cells.

Clonogenic assay

NB4 cells stably expressing anti-miR-181a, anti-miR-181b, or an unspecific control sequence were seeded in a density of 1 × 10^3 cells/mL in methylcellulose-based media (MethoCult H4230; StemCell Technologies) as triplicates according to the manufacturer’s instructions. Replating was done after 6 days and repeated three times. Colony numbers were evaluated after each plating by standard criteria. For colony size measurement, pictures were taken randomly from each condition and a total number of 135 colonies were measured by using ImageJ software.

miRNA and mRNA detection by quantitative real-time PCR

Total RNA was extracted using TRIzol. miRNA quantification was performed as previously described by using hasa-miR-181a and hasa-miR-181b primer sets or mmu-miR-181a and mmu-miR-181b-1 primer sets (Applied Biosystems Inc.). Normalization was done by measuring RNU6B (U6) expression and small nuclear RNA135 (snoR135) expression. mRNA amplification was performed as previously described by using GAPDH expression for normalization (11). Primer sequences are provided in Supplementary Table S3. PCR reactions were performed in a MyiQ2 Two-Color Real-Time PCR Detection System (Bio-Rad).

Immunoblot analysis

Immunoblot analysis was performed as previously described (11). Following antibodies were used: mouse monoclonal and polyclonal antibody anti-RASSF1A for analyzing human cell lines and primary patient cell samples, rabbit polyclonal antibody anti-RASSF1A for analyzing mouse bone marrow samples (both Abcam) and rabbit polyclonal antibody anti-RARα, rabbit polyclonal antibody anti-cyclin D1 and rabbit polyclonal antibody anti-p53 (Santa Cruz Biotechnology). Rabbit polyclonal antibodies anti-β-tubulin and anti-GAPDH were used for normalization.
(both Santa Cruz Biotechnology). Immunodetection was performed with WesternSure Chemiluminescent Substrate (LI-COR Biosciences). Band intensities were quantified using ImageJ software (NIH, Bethesda, MD).

Flow cytometry
Cell differentiation was evaluated by direct immunofluorescent staining using phycoerythrin (PE)-conjugated mouse anti-human CD11b/Mac-1, allophycocyanin (APC)-conjugated mouse anti-human CD11b/Mac-1 (BD Biosciences), and APC-conjugated mouse anti-CD114 (GCSF-R; Biolegend) cell surface myeloid-specific antigens. Apoptosis was measured with a PE Annexin V Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer’s instructions. Cell cycle was measured by performing ethanol fixation of cells followed by RNase A digestion and propidium iodide staining of DNA. A minimum of 10,000 events were collected for each sample by a FACScan flow cytometer (Becton Dickinson) using CellQuest software for data acquisition and CytoLogic software for data analysis.

Statistical analysis
We used the Student t test to determine statistical significance of experimental results. A P value of 0.05 or less was considered significant (*) and a P value of 0.01 or less were considered as highly significant (**). The results were represented as the median ± SD from three independent experiments for cell line experiments and from two independent reverse transcription and quantitative PCR (qPCR) analysis for primary cell samples.

Results
ATRA treatment represses miR-181 family member expression in APL cell line and in APL patients
Several studies show the modulation of miRNA pattern upon ATRA treatment (10, 14, 19). We analyzed miR-181 family member expression (miR-181a-d) 24 hours after ATRA treatment and observed a significant downregulation of all miR-181 family members (Fig. 1A). The genomically clustered miR-181a and miR-181b show similar expression levels, whereas miR-181c and miR-181d, also organized in a genomic cluster, were differentially expressed (20). miR-223 expression was used as experimental control and showed a 2-fold upregulation (Fig. 1B).

Furthermore, we induced granulocytic differentiation by ATRA in NB4 cells and the non-APL cell lines U937 and HL60. Differentiation was confirmed by CD11b measurement (Fig. 1C, E, and G). NB4 cells showed a significant reduction of miR-181a/b expression over time (Fig. 1D). No significant change of miR-181a/b expression could be observed in U937 (Fig. 1F) and HL60 cells (Fig. 1H).

In clinical APL therapy, ATRA is used in combination with anthracyclines, cytarabines, and arsenic trioxide. To test their effects on miR-181a/b expression, NB4 cells were treated with anthracycline (doxorubicin) with or without cytarabine (ara-c) or arsenic trioxide (As2O3) and the appreciate controls for 24 hours. The results showed no repression of miR-181a/b expression after treatment with cytarabine or arsenic trioxide in contrast to ATRA, which strongly reduced the miRNA expression (Fig. 1I and J). 7-Aminoactinomycin D (AAD)/Annexin V measurement revealed strong induction of apoptosis by cytarabine and As2O3.

miR-181a/b Targets RASSF1A in APL
We used the Student t test to determine statistical significance of experimental results. A P value of 0.05 or less was considered significant (*) and a P value of 0.01 or less were considered as highly significant (**). The results were represented as the median ± SD from three independent experiments for cell line experiments and from two independent reverse transcription and quantitative PCR (qPCR) analysis for primary cell samples.

miRNA-181a/b are induced by the oncogenic fusion protein PML/RARα in vivo and in vitro
The oncogenic fusion protein PML/RARα is known to regulate a huge number of different genes (16, 18, 21). To show the regulatory influence of PML/RARα on miR-181a/b expression, we induced PML/RARα protein in U937-PR9 cells with ZnSO4. miR-181a/b expression was immediately upregulated upon PML/RARα induction (Fig. 2A). The control cell line U937-PC18 showed no significant change in miRNA expression after ZnSO4 application (Fig. 2B). In addition, we analyzed bone marrow samples from PML/RARα knock-in mice and wild-type animals (C57Bl/6-WT; ref. 17). We observed a significant enhanced expression of murine miR-181a/b in PML/RARα knock-in mouse samples in comparison to wild-type samples (Fig. 2C). Furthermore, we analyzed bone marrow samples from patients with different AML subtypes and blood cell samples from healthy donors. The results showed significantly higher miR-181a/b expression values in APL patient samples than in the samples with normal karyotype, whereas all other analyzed samples showed no significant miRNA expression change (Supplementary Table S2 and Fig. 2D).

The miR-181a/b cluster is necessary for proliferation, induces apoptosis, and inhibits granulocytic differentiation of APL cells
To address the role of miR-181a/b in APL in detail, we stably knocked down both miRNAs in NB4 cells by using miR-ZIP-lentiviral particles. Knockdown efficiency was verified by qPCR (Fig. 3A). The PML/RARα fusion protein promotes cell survival (4). To evaluate a potential role for miR-181a and miR-181b in this process, we examined an apoptosis assay. Knockdown of miR-181a and miR-181b results in significantly increased apoptosis (Fig. 3B). According to this, p53 protein was induced in the miR-ZIP-181a and -181b expressing cells compared with the control cells (Supplementary Fig. S2). Furthermore, in a replating assay, we could show strongly reduced colony size and colony-forming capacity of NB4 cells after knockdown of miR-181a and miR-181b (Fig. 3C and D). According to this, the proliferation rate of miR-ZIP-181a- or miR-ZIP-181b-expressing cells was significantly reduced (Fig. 3E).

To investigate the influence of miR-181a/b on ATRA-induced differentiation of APL cells, we transiently transfected NB4 cells with miR-181a- and miR-181b-specific mimics and control mimics. Granulocytic differentiation was induced by ATRA 24 hours after transfection. CD11b expression was significantly decreased after overexpression of one of the miRNAs in comparison to the control 48 hours after transfection. The effect was slightly increased by simultaneous transfection of miR-181a and
In APL, miR-181 family member expression is repressed by ATRA in vitro and in vivo. A and B, qPCR for miR-181 family member and miR-223 expression 24 hours after ATRA treatment of NB4 cells. C, E, and G, FACS for CD11b expression in NB4 (C), U937 (E), and HL60 cells (G) 72 hours after ATRA application. D, F, and H, qPCR for miR-181a/b in NB4 (D), U937 (F), and HL60 cells (H) after ATRA application at indicated time points. I and J, qPCR for miR-181a/b in NB4 cells treated with As2O3, doxorubicin (doxo) w/o cytarabine or ATRA for 24 hours. K and L, qPCR for miR-181a/b in bone marrow samples from APL patient at diagnosis time point (newly diagnosed APL) and during ATRA treatment (APL during ATRA-based therapy). n.s., not significant. *, $P \leq 0.05$; **, $P \leq 0.01$. 

Figure 1.
Figure 2. PML/RARα induces miR-181a and miR-181b expression *in vitro and in vivo*. A and B, U937-PR9 and the control cell line U937-PC18 were treated with ZnSO4 or H2O (vehicle). qPCR for miR-181a/b was performed at indicated time points. Western blotting for RARα and PML/RARα protein upon ZnSO4 application in PR9 and PC18 cells (left). C, qPCR for murine miR-181a and miR-181b (mmu-miR-181b-1) in 5 PML/RARα knock-in mice (C57BL/6-mCG^+/PR^) and 5 wild-type mice (C57BL/6-WT). Data represents the dCt values. D, qPCR for miR-181a/b expression in bone marrow samples from patients with AML, with indicated subtypes and healthy donors. n.s., not significant. *, P < 0.05; **, P < 0.01.
miR-181b mimics (Fig. 3F). In addition, qPCR showed a strong decrease of ATRA induced C/EBPβ and granulocyte colony-stimulating factor (GCSF) receptor mRNA expression 48 hours after miR-181a and miR-181b mimic transfection in comparison to the control (Fig. 3G).

The tumor suppressor RASSF1A is a direct target of the miR-181a/b cluster

We hypothesized that miR-181a and miR-181b are involved in formation of the PML/RARα-induced oncogenic transformation by targeting differentiation-required genes. By computational analysis using miRNA target prediction programs such as Target Scan (http://www.targetscan.org), we identified the tumor suppressor Ras association domain family member 1 isoform A (RASSF1A) as a putative target of the miR-181 family (Fig. 4D). RASSF1A is a well-characterized tumor suppressor that is epigenetically suppressed by promoter hypermethylation in a wide range of tumors (22). In APL, no case is reported until now (23). We evaluated RASSF1A protein in NB4 cells by Western blotting. The data showed a significant increase of RASSF1A protein upon ATRA treatment over time (Fig. 4A). Interestingly, RASSF1A protein showed no upregulation in NB4 cells upon induction of apoptosis by arsenic trioxide (Fig. 4B and Supplementary Fig. S1A). The 3’UTR of RASSF1A harbors three potential miR-181–binding sites (Fig. 4C). To analyze direct binding of miR-181a and miR-181b to the 3’UTR of RASSF1A, we generated a luciferase construct containing the complete 3’UTR of RASSF1A and mutated the binding sites (Fig. 4D and E). Reporter assay showed repression of luciferase activity after miR-181a and miR-181b mimic transfection in comparison to the control. Mutation of the miR-181–binding sites resulted in the recovery of luciferase activity and revealed direct binding of miR-181a/b to the 3’UTR of RASSF1A (Fig. 4F).

To validate our finding that RASSF1A is a direct target of miR-181a/b, we analyzed RASSF1A protein after miR-181a and miR-181b mimic transfection in U937 cells and observed repression of RASSF1A protein 24 hours and much stronger 48 hours after transfection (Fig. 4G). In addition, RASSF1A protein was increased after miR-ZIP–mediated knockdown of miR-181a and miR-181b in NB4 cells compared with the control cells (Fig. 4H).

RASSF1A protein is specifically suppressed in APL

RASSF1A is shown to be an important regulator of cell differentiation in a wide range of cell types (22) by exercising its functions as a modulator of two pathways commonly deregulated in cancer, apoptosis, and cell cycle (24, 25). Therefore, we hypothesize that RASSF1A could function as tumor suppressor in APL, where PML/RARα causes oncogenic transformation by deregulating cell cycle and apoptosis (4). Western blot analysis of bone marrow samples from patients with different AML subtypes and blood cell samples from healthy donors showed significantly lower amounts of RASSF1A protein in patients with APL compared with patients with AML with normal karyotype (Supplementary Table S2 and Fig. 5A). No significant change in RASSF1A protein levels could be observed in other AML subtypes and in non-AML samples. Interestingly, RASSF1A mRNA exhibited a heterogeneous distribution and especially no reduction in t(15;17) (Fig. 5B). Furthermore, we observed significantly lower RASSF1A protein levels in PML/RARα knock-in mice (C57Bl/6-mCg-C17/P) compared with wild-type animals (C57Bl/6-WT) (Fig. 5C).

RASSF1A is essential for ATRA-induced granulocytic differentiation and induces apoptosis in APL

To verify the hypothesized differentiation-associated function of RASSF1A, we used a set of four constructs encoding different shRNA sequences specific for RASSF1A mRNA to generate a stable NB4 RASSF1A-knockdown cell line. The knockdown efficiency was confirmed by qPCR and showed a significant reduction of RASSF1A mRNA to 0.2-fold in the RASSF1A shRNAs expressing cells in comparison to the cells expressing an unpecific shRNA (Fig. 6A). ATRA-induced CD11b expression and GCSF-R expression were strongly repressed in RASSF1A shRNA-expressing cells in comparison to the control cells (Fig. 6B). Transient overexpression of RASSF1A was performed to point out the tumor-suppressive function of RASSF1A in APL. Apoptosis assay displayed a strong increase in the Annexin V–positive cell population 24 hours after pcDNA3.1-RASSF1A transfection in comparison to the empty vector transfection (Fig. 6C).

miR-181a/b and RASSF1A modulate differentiation in APL via regulation of cell cycle

ATRA-induced cell growth arrest and terminal differentiation of APL blasts involves downregulation of cyclin D1 (26). To confirm the ATRA-dependent downregulation of cyclin D1 in APL cells, we performed Western blotting 48 hours after ATRA stimulation of NB4 cells and observed a decrease in cyclin D1 protein (Fig. 7A). RASSF1A is also able to induce cell-cycle arrest by inhibition of cyclin D1 accumulation (24). Overexpression of RASSF1A by pcDNA3.1/RASSF1A resulted in a complete repression of cyclin D1 protein in NB4 cells after 24 hours in comparison to the control vector transfection (Fig. 7B). We also performed cell-cycle analysis 24 hours after ATRA treatment of RASSF1A shRNA-expressing NB4 cells and observed that shRNA-mediated knockdown of RASSF1A leads to a marked reduction in G1–G0 phase and an increase in the S- and G2 phase in comparison to the control shRNA-expressing cells (Fig. 7C). In addition, we analyzed cell cycle in miR-ZIP-181a- and miR-ZIP-181b–expressing NB4 cells. The results showed that knockdown of miR-181a and miR-181b reduces S- and G2 phase and increases G1–G0 phase as well.

Figure 3.

In APL, the miR-181a/b cluster is necessary for cell proliferation, induces apoptosis, and inhibits granulocytic differentiation. A, qPCR for miR-181a/b in NB4 cells stably expressing miR-ZIP-181a, miR-ZIP-181b, or miR-ZIP control sequences. B, apoptosis assay in miR-ZIP-181a, miR-ZIP-181b, and miR-ZIP control expressing NB4 cells. C, replating assay of miR-ZIP-181a/b or miR-ZIP control expressing NB4 cells. D, replating of bone marrow samples from patients with APL compared with patients with AML with normal karyotype. E, cell growth curve of miR-ZIP-181a/b or miR-ZIP control expressing NB4 cells. F, FACS for ATRA-induced CD11b expression 48 hours after miR-181a mimic, miR-181b mimic, or control mimic transfection of NB4 cells. The values indicate the amount of CD11b-positive cells (%) for each transfection condition. G, qPCR for C/EBPβ and GCSF-R mRNA in miR-181a, miR-181b, or control mimic transfected NB4 cells 48 hours after transfection. *, P ≤ 0.05; **, P ≤ 0.01.
Bräuer-Hartmann et al.

**A**

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>ATRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>48 h</td>
<td>0.9</td>
<td>1.4</td>
</tr>
<tr>
<td>72 h</td>
<td>2.9</td>
<td>2.9</td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>AsA-O3 1 mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>48 h</td>
<td>1</td>
<td>0.8</td>
</tr>
</tbody>
</table>

**C**

![Diagram of RASSF1A ORF and miR-181 binding sites](image)

**D**

![Diagram of pGL3 control vector and pGL3/3'UTR RASSF1A vectors](image)

**E**

<table>
<thead>
<tr>
<th>Position</th>
<th>Sequence</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'</td>
<td>UUUUUUAUGAAGGGCGAGGAUGUA</td>
<td>RASSF1 3' UTR WT (position 1: 671–677)</td>
</tr>
<tr>
<td>3'</td>
<td>...ACUUACA...</td>
<td>hsa-miR-181</td>
</tr>
<tr>
<td>5'</td>
<td>UUUUUUAUGAAGGGCGACACUCUC</td>
<td>RASSF1 3' UTR mutated (position 1: 671–677)</td>
</tr>
<tr>
<td>3'</td>
<td>...ACUUACA...</td>
<td></td>
</tr>
<tr>
<td>5'</td>
<td>GGGCGAGGGUCUGUGAAGGAUGUG</td>
<td>RASSF1A 3' UTR WT (position 2: 587–593)</td>
</tr>
<tr>
<td>3'</td>
<td>...ACUUACA...</td>
<td>hsa-miR-181</td>
</tr>
<tr>
<td>5'</td>
<td>GGGCGAGGGUCUGUGAACCUAUG</td>
<td>RASSF1A 3' UTR mutated (position 2: 587–593)</td>
</tr>
<tr>
<td>3'</td>
<td>...ACUUACA...</td>
<td></td>
</tr>
<tr>
<td>5'</td>
<td>AAGGGUUGUGAAGCUUGAAUGUG</td>
<td>RASSF1 3' UTR WT (position 3: 392–398)</td>
</tr>
<tr>
<td>3'</td>
<td>...ACUUACA...</td>
<td>hsa-miR-181</td>
</tr>
<tr>
<td>5'</td>
<td>AAGGGUUGUGAAGCUUCCAGCGG</td>
<td>RASSF1 3' UTR mutated (position 3: 392–398)</td>
</tr>
</tbody>
</table>

**F**

![Graph showing RLU values for U937](image)

**G**

![Graph showing RASSF1A expression](image)

**H**

![Graph showing RASSF1A expression](image)
as sub-G₁ phase (Fig. 7D). To show the regulatory impact of miR-181a and miR-181b on cyclin D1, we transiently transfected miR-181a and miR-181b mimics in U937 cells. Western blotting showed an elevated protein level of cyclin D1 48 hours after single or combined miR-181a and miR-181b mimic transfection compared with the control (Fig. 7E). To prove that cyclin D1

Figure 5.
RASSF1A protein is specifically suppressed by PML/RARα. A, Western blotting for RASSF1A protein in bone marrow samples of patients with different AML subtypes and cell samples from nonleukemic patients (healthy donors). Bars in the diagram show the median of the normalization ratio for each patient group. Western blot (right) analysis of RASSF1A protein in each analyzed patient sample, with the corresponding normalization ratio below. B, qPCR for RASSF1A mRNA expression in AML patient samples. C, Western blotting for RASSF1A protein in bone marrow samples from PML/RARα knock-in mice (C57Bl/6-mCG⁺/PR) and wild-type mice (C57Bl/6-WT; right). The number of mice (n) or patients (n) in each group is shown below the bars. *, P ≤ 0.05.
protein increment upon miR-181a and miR-181b mimic transfection is mediated by repression of RASSF1A, we cotransfected miR-181a and miR-181b mimics and the corresponding pcDNA3.1/control vector in NB4 cells. Western blotting 48 hours after transfection revealed a significant repression of cyclin D1 protein when RASSF1A is expressed lacking a 3’UTR in the presence of miRNA mimics (Fig. 7F).

Discussion

An increasing number of studies have shown the importance of miRNAs in the formation and maintenance of leukemia. In this report, we demonstrate that ATRA is able to significantly downregulate the expression of the whole miR-181 family in APL (Fig. 1A). We show the constant downregulation of the miR-181a/b cluster upon ATRA treatment over time in APL in vitro and in vivo (Fig. 1D, K, and L). Furthermore, we demonstrate that cytostatics and arsenic trioxide, which are typically used in APL therapy and are predominately inducers of apoptosis, do not affect miR-181a/b expression (Fig. 11 and I). These results expand and confirm previous observations (10, 14–16) and suggest a specific role for the miR-181 family in the response to ATRA in APL. Diverse publications illustrate the expression pattern and define multiple functions for miR-181a and miR-181b in hematopoiesis and leukemia, whereas miR-181c and miR-181d are less described (8, 27–31). The fact that ATRA leads to the degradation of PML/RARα and thereby changes gene expression, assuming that miR-181a/b expression is dependent on PML/RARα (1). We followed miR-181a/b expression upon ATRA treatment of the non-APL cell lines U937 and HL60. Both cell lines respond to ATRA but show no significant change in miR-181a/b expression (Fig. 1E–H). This observation substantiates the proposed PML/RARα dependency of miR-181a/b expression.

The miR-181a/b cluster has been shown to be upregulated in patients with AML with C/EBPα mutations who have a favorable prognosis and have also shown to be associated with favorable outcome in patients with cytogenetically normal AML and cytogenetically abnormal AML (32–34). Combining these data, high expression of miR-181a and miR-181b occurs in combination with a favorable outcome of AML. In APL, a combination of ATRA and arsenic trioxide therapy generates a complete remission rate (CR) of more than 90% (35). Our observation that the miR-181a/b cluster is highly expressed in
APL and significantly downregulated upon ATRA treatment in vitro and in vivo points to a role for the miRNA cluster as prognostic marker in t(15;17).

Besides its function as transcriptional repressor (2), PML/RARα is also able to induce transcription, whereas this effect seems to be indirect due the sequestration of corepressors (36). In this study, we demonstrate the PML/RARα-dependent upregulation of miR-181a/b in PR9 cells and in PML/RARα knock-in mice (Fig. 2A–C). In addition, we show significantly higher expression of the miR-181a/b cluster in bone marrow samples from patients with APL (Fig. 2D). These results are reinforced by data from Li and colleagues (31) and Jongen-Lavrencic and colleagues (32).
together, to the best of our knowledge, we are the first to show PML/RARA-dependent upregulation of the miR-181a/b cluster in AML. Because PML/RARA has no direct binding site in the promoter region of the miR-181a/b cluster (16), the transcriptional induction has to occur indirectly. The exact mechanism how the miRNA cluster is regulated is still unknown and has to be investigated in further experiments.

Diverse publications describe miR-181 family members as either oncogenes or tumor suppressors in various cancers depending on tissue type (37–40). The fact that miR-181a/b expression is significantly high in t(15;17), assuming that both miRNAs are involved in the formation of the oncogenic phenotype caused by PML/RARA. Our functional studies show that ectopic expression of miR-181a and miR-181b effectively blocks ATRA-induced granulocytic differentiation (Figs. 3F and G) and that inhibition of the miR-181a/b cluster effectively represses cell proliferation and induces apoptosis in APL cells (Fig. 3A–E). In contrast to data from Hickey and colleagues (33) and Li and colleagues (34) who assign miR-181a as an anti-leukemic miRNA in AML, our results show an oncogenic function for the miR-181a/b cluster in APL. This is supported by recently published data from Su and colleagues, which showed that miR-181a blocks myeloid differentiation of HL60 and CD34+ hematopoietic stem/progenitor cells (41).

In cancer, oncogenic miRNAs exercise their function by targeting tumor suppressors (42, 43). In our study, we identify the known tumor suppressor RASSF1A (Ras association domain family member 1 isoform A) as a direct target of the miR-181a/b cluster in APL. miR-181 family members are the only miRNAs that have three conserved binding sites in the 3′UTR of RASSF1A (Fig. 4A and E). RASSF1A has been found to be epigenetically inactivated in a variety of cancers by promoter hypermethylation, and reintroduction of RASSF1A in RASSF1A-deficient tumor cells leads to the reduction of tumorigenicity (22, 44). Because there is no RASSF1A promoter hypermethylation in APL, there must be other mechanisms how the tumor suppressor is inactivated (23, 45). In this study, we show the upregulation of RASSF1A protein upon ATRA treatment in NB4 cells while miR-181a/b expression is decreasing (Figs. 1A, D, K, and L and 4A). In addition, we demonstrate that RASSF1A protein is not regulated by arsenic trioxide, which also does not affect miR-181a/b expression (Fig. 4B, Fig. 1I and J). Finally, we prove by luciferase assay that repression of RASSF1A protein occurs via direct binding of miR-181a and miR-181b to its 3′UTR (Fig. 4F), which has also been shown in hepatocellular cancer stem cells by Meng and colleagues (46). Our luciferase assay data in combination with our data from knockdown and overexpression experiments of miR-181a and miR-181b firstly show the direct repression of RASSF1A translation by the miR-181a/b cluster in the background of APL (Fig. 4F–H). Furthermore, we observed the APL-specific inverse correlation of miR-181a/b expression and RASSF1A protein, which could not be seen in the other analyzed AML subgroups and healthy donors. The proposed specificity of RASSF1A suppression by a PML/RARA-dependent mechanism is supported by protein expression data in AML patient samples and in PML/RARA knock-in mice, which show reduced RASSF1A protein levels when the miR-181a/b cluster is highly expressed (Figs. 2C and D and 5). In addition, mRNA expression analysis of RASSF1A in AML patient samples and healthy donors substantiates the suggested regulation mechanism via miR-181a/b (Fig. 5B).

These findings are supported by a recently published work by Zare-Abdollahi and colleagues (47).

Until now, no function for RASSF1A in APL or granulocytic differentiation has been shown. In this study, we describe RASSF1A as an essential component of the ATRA-induced granulocytic differentiation network in APL. Enforced expression of RASSF1A leads to enhanced apoptosis of NB4 cells, which confirms the tumor-suppressive function of RASSF1A in APL (Fig. 6C). In addition, reduced granulocytic differentiation of NB4 cells in consequence to RASSF1A knockdown supports the proposed differentiation associated function of RASSF1A (Fig. 6B). It was shown that RASSF1A is able to effectively prevent G1–S phase transition by blocking cyclin D1 accumulation (24) and to induce apoptosis (48). In APL, ATRA induces APL cell differentiation into mature granulocytes and results in cell apoptosis (26). This process involves the sequential regulation of cell-cycle regulatory proteins, such as cyclin D1, which promotes G1–S progression (49). In our study, we confirm the ATRA-induced repression of cyclin D1 in APL cells (Fig. 7A). We also show that overexpression of RASSF1A leads to a dramatic repression of cyclin D1 protein and that knockdown of RASSF1A promotes cell-cycle progression in APL (Fig. 7B and C). Furthermore, we could demonstrate that RASSF1A is the key mediator for miR-181a- and miR-181b-mediated induction of cyclin D1 accompanied by cell-cycle progression in APL (Fig. 7E and F). Finally, in contrast to ATRA, arsenic trioxide, which induces apoptosis in APL, does not affect miR-181a/b expression and RASSF1A protein. This correlates with the finding that arsenic trioxide does not downregulate cyclin D1 protein (50). On the basis of these findings, we claim RASSF1A as an important factor in the granulocytic differentiation, which prevents cyclin D1 accumulation, cell-cycle progression, and promotes differentiation upon ATRA treatment in APL blasts.

In summary, our study highlights the clustered miR-181a and miR-181b as important factors in the PML/RARA-associated APL. To the best of our knowledge, we are the first describing the miR-181a/b target RASSF1A as an essential member of the ATRA-induced granulocytic differentiation network in APL (Fig. 7G). Both miRNAs, transcriptional induced by PML/RARA, lead to translational repression of the tumor suppressor RASSF1A. Its function is restored trough repression of miR-181a/b expression by ATRA-induced degradation of PML/RARA, which prevents accumulation of cyclin D1 and induces cell-cycle arrest, which results in granulocytic differentiation of APL blasts. Our data reveal a mechanism of tumor suppressor inhibition by a microRNA cluster that seems to be highly specific for APL. Finally, manipulation of miR-181a/b could offer novel treatment strategies in PML/RARA-associated APL.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: D. Bräuer-Hartmann, C. Behre
Development of methodology: D. Bräuer-Hartmann, J.-U. Hartmann, C. Behre
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Bräuer-Hartmann, J.-U. Hartmann, M.V. Verga Falzacappa, P.G. Pellicci, G. Behre
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Bräuer-Hartmann, J.-U. Hartmann, A.A. Wurm, C. Kaiznerke, D. Niederwieser, C. Behre
Writing, review, and/or revision of the manuscript: D. Bräuer-Hartmann, D. Gerloff, C. Kaiznerke, C. Müller-Tidow, D.G. Tenen, D. Niederwieser, G. Behre
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Bräuer-Hartmann, J.-U. Hartmann, A.A. Wurm, D. Gerloff, C. Katzerke, D.G. Tenen, D. Niederwieser, G. Behre
Study supervision: D. Niederwieser, G. Behre

Acknowledgments
The authors thank R. Dammann for providing pGL3 1/RASSF1A expression construct.

Grant Support
This study was supported by grants from DFG (German Research Foundation, BE 2042/7-1, BE 2042/12-1), Deutsche José Carreras Stiftung E.V., and Translational Centre for Regenerative Medicine Leipzig to G. Behre and Deutsche José Carreras Stiftung E.V. to D. Bräuer-Hartmann and the National Institute of Health (CA66996 and HL112719) to D.G. Tenen.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 1, 2014; revised April 27, 2015; accepted May 12, 2015; Published OnlineFirst June 3, 2015.

References

miR-181a/b Targets RASSF1A in APL.


PML/RARα-Regulated miR-181a/b Cluster Targets the Tumor Suppressor RASSF1A in Acute Promyelocytic Leukemia

Daniela Bräuer-Hartmann, Jens-Uwe Hartmann, Alexander Arthur Wurm, et al.

Cancer Res  Published OnlineFirst June 3, 2015.

Updated version  Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-14-3521

Supplementary Material  Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2015/06/03/0008-5472.CAN-14-3521.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.