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PML/RAR α -regulated miR-181a/b-cluster targets the tumor suppressor RASSF1A in Acute Promyelocytic Leukemia

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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 key event in the drug response. We found that miR-181a/b expression was activated by the PML/RAR α 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. 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'UTR. 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. Lastly, RASSF1A levels were reduced in PML/RAR α knock-in mice and APL patient samples. Taken together, our results define miR-181a and miR-181b as oncomiRs in PML/RAR α -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 (RAR α), (1,2). The most frequent

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translocation fuses the RAR α with the promyelocytic leukemia protein (PML) gene (3). At physiological levels of retinoids, the PML/RAR α fusion protein causes block of differentiation and neoplastic transformation by disrupting the function of PML and repressing transcription of genes regulated by RAR α (2,4,5). Pharmacological doses of retinoids can overcome this block, lead to the expression of granulocytic specific transcription factors like C/EBP β (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 (miRNAs) are endogenous, non-protein coding small RNAs which play critical roles in the post-transcriptional 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 ATRA-induced differentiation (14-16). By analyzing APL and AML patient samples as well as PML/RAR α knock-in mice, we demonstrated that miR-181a and miR-181b display a very specific PML/RAR α -dependency *in vivo*. Furthermore, we revealed that miR-181a and miR-181b are involved in the formation of the PML/RAR α 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.

Material 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) 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/RAR α knock-in mice (C57BI/6-mCG^{+PR}) in which PML/RAR α is expressed under the control of the murine cathepsin G gene (C57BI/6-mGC^{+PR}) and 5 wild type mice (C57BI/6-WT) were obtained from Pier Giuseppe Pelicci (IFOM-EIO, Milan; Italy);(17).

Cells, reagents, and transfections

NB4, HL60 and U937 cells were cultured under standard conditions. For differentiation, cells were induced with 10^{-6} M ATRA (Sigma-Aldrich, St. Louis, MO, USA) and as control DMSO. For differentiation of microRNA mimic transfected and RASSF1A shRNA expressing NB4 cells 10^{-7} M ATRA were used. Arsenic trioxide (As_2O_3 ; Sigma-Aldrich) were used at 1 μM . As control 1% HCl solution was used. Doxorubicin (Selleckchem; Houston, TX, USA), solved in DMSO, were applied at 0.01 $\mu\text{g}/\text{ml}$ and cytarabine at 0.05 μM . Transfection of microRNA mimics (Dharmacon, Lafayette, CO, USA) and pcDNA3.1-vectors were done by using Amaxa technology (Lonza, Basel, Switzerland) according to the manufacturer's instructions. The transfection efficiency was 30 – 40%. U937-PR9 cells, which carry the PML/RAR α cDNA under the control of metallothionine promoter, and the control cells U937-PC-18, which carry the empty vector, were cultured and the expression of the PML/RAR α fusion protein was induced as previously described (18).

DNA constructs, cloning and mutagenesis

For luciferase assay RASSF1A 3'-UTR was inserted in the pGL3-luciferase reporter control vector downstream of the luciferase encoding region (Promega, Fitchburg, Wisconsin, USA). MiR-181 binding site positions in the 3'UTR were taken from the microRNA target data base targetscan (www.targetscan.org). The RASSF1A 3'UTR (Acc. No. [NM_007182.4]) was amplified from cDNA of NB4 cells treated with 1 μM ATRA for 48 h using primer pairs, which generate a XbaI restriction enzyme recognition site at the 3' and 5' end of the amplified DNA product. The following primer pairs were used: RASSF1A 3'UTR forward, 5'-GTCTAGACCTCTTGTACCCCCAGGTGG-3'; RASSF1A 3'UTR reverse, 5'-GTCTAGAGAGGATCTTGAAATCTTTATTGAG-3'. The purified DNA-fragment and the pGL3-luciferase reporter control vector were digested with XbaI and fused in a T4 ligase reaction (Invitrogen, Carlsbad, CA, USA). Mutagenesis of miR-181 binding sites was done with the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) 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 (pGL3-control vector, pGL3/3'UTR-RASSF1A and pGL3/3'UTR-RASSF1A-mutated), 0.1 μg of Renilla construct (pRL) and 1 μM 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 Bioscience (Mountain View, CA, USA) and p-RFP-CB-shLenti-vectors were purchased from Origene (Rockville, MD, USA). MiR-ZIP-lentiviral particles were produced according to the manufacturer's instructions.

NB4 cells were infected 2 times within 48h with 10 μ l of PEG-itTM-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 4 different shRNA-vectors and the control-shRNA-vector accordingly 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 1×10^4 cells/ml. Proliferation rate was ascertained by cell counting in a Neubauer camber using Trypan blue-staining for excluding death 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 3 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 hsa-miR-181a and hsa-miR-181b primer sets or mmumiR-181a and mmu-miR-181b-1 primer sets (Applied Biosystems Inc., CA, USA). Normalization was done by measuring RNU6B (U6) expression and small nuclear RNA135 (snoR135) expression. mRNA amplification was performed as previously described by using glyceraldehyde-3-phosphate dehydrogenase (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 (BIORAD, Hercules, CA, USA).

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-RASSF1 for analyzing mouse bone marrow samples (both Abcam, Cambridge, UK) and rabbit polyclonal antibody anti-RAR α , rabbit polyclonal antibody anti-Cyclin D1 and rabbit polyclonal antibody anti-p53 (Santa Cruz Biotechnology, Dallas, Texas, USA). 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 bioscience, Lincoln, NE, USA). Band intensities were quantified using Image J software (National Institutes of Health).

Flow cytometry

Cell differentiation was evaluated by direct immunofluorescence staining using Phycoerythrin (PE) conjugated mouse anti-human CD11b/Mac-1, Allophycocyanin (APC) conjugated mouse anti-human CD11b/Mac-1 (BD Bioscience, Franklin Lakes, NJ, USA) and APC conjugated mouse anti-CD114 (GCSF-R; Biolegend, San Diego, CA, USA) cell-surface myeloid-specific antigens. Apoptosis was measured with a PE Annexin V Apoptosis Detection Kit I (BD Bioscience) 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 Cyflogic 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 plus or minus Standard deviation (SD) from 3 independent experiments for cell line experiments and from 2 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 microRNA pattern upon ATRA-treatment (10,14,19). We analyzed miR-181 family member expression (miR-181a-d) 24h after ATRA-treatment and observed a significantly 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,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; doxo) with or without cytarabine (ara-c) or arsenic trioxide (As₂O₃) and the appropriate controls for 24h. The results showed no repression of miR-181a/b expression after treatment with cytostatics or arsenic trioxide in contrast to ATRA which strongly reduced the microRNA expression (Fig 1I, J). 7-AAD/Annexin V measurement revealed strong induction of apoptosis by cytostatics and As₂O₃. CD11b cell

surface marker and C/EBP β mRNA were slightly and only temporary induced by As₂O₃ or cytostatics compared to ATRA which strongly induced granulopoiesis (Supplementary Fig. S1). Furthermore, we used samples from APL patients which received a combination of ATRA with chemotherapy to investigate the expression of miR-181a/b. Analysis was performed at time point of diagnosis and at one time point during therapy (Supplementary Table S1). In all 6 analyzed patients we observed a highly significant repression of miR-181a/b expression in consequence to ATRA-based therapy (Fig. 1K,L).

MiRNA-181a/b are induced by the oncogenic fusion protein PML/RAR α *in vivo* and *in vitro*

The oncogenic fusion protein PML/RAR α is known to regulate a huge number of different genes (16,18,21). To show the regulatory influence of PML/RAR α on miR-181a/b expression, we induced PML/RAR α protein in U937-PR9 cells with ZnSO₄. MiR-181a/b expression was immediately upregulated upon PML/RAR α induction (Fig. 2A). The control cell line U937-PC18 showed no significant change in microRNA expression after ZnSO₄ application (Fig. 2B). Additionally, we analyzed bone marrow samples from PML/RAR α knock-in mice and wild type animals (C57Bl/6-WT); (17). We observed a significant enhanced expression of murine miR-181a/b in PML/RAR α 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 compared to the samples with normal karyotype whereas all other analyzed samples showed no significant microRNA expression change (Supplementary Table S2; 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 microRNAs in NB4 cells by using miR-ZIP-lentiviral particles. Knock down efficiency was verified by qPCR (Fig. 3A). The PML/RAR α 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 to 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 knock down of miR-181a and miR-181b (Figure 3C and D). According to this, the proliferation rate of miR-ZIP-181a or -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 24h postransfection. CD11b expression was significantly decreased after overexpression of one of the microRNAs in comparison to the control 48h after transfection. The effect was slightly increased by simultaneous transfection of miR-181a and miR-181b mimics (Fig. 3F). In addition, qPCR showed a strong decrease of ATRA induced C/EBP β and GCSF-receptor

mRNA expression 48h 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/RAR α -induced oncogenic transformation by targeting differentiation-required genes. By computational analysis using microRNA 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 which 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 blot. 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; Supplementary Fig. S1A). The 3'UTR of RASSF1A harbors 3 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,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 24h and much stronger 48h after transfection (Fig. 4G). Additionally, RASSF1A protein was increased after miR-ZIP-mediated knockdown of miR-181a and miR-181b in NB4 cells compared to 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/RAR α 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 significant lower amounts of RASSF1A protein in APL patients compared to AML patients with normal karyotype (Supplementary Table S2; 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 significant lower RASSF1A protein levels in PML/RAR α knock-in mice (C57BI/6-mCG^{+/PR}) compared to wild type animals (C57BI/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 4 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 unspecific shRNA (Fig. 6A). ATRA-induced CD11b expression and G-CSF-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 tumorsuppressive function of RASSF1A in APL. Apoptosis assay displayed a strong increase in the Annexin V positive cell population 24h 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 blot 48h 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 24h in comparison to the control vector transfection (Fig. 7B). We also performed cell cycle analysis 24h 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). Additionally, 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 as SubG1-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 blot showed an elevated protein level of cyclin D1 48h after single or combined miR-181a and miR-181b mimic transfection compared to the control (Fig. 7E). To prove that cyclin D1 protein increasment upon miR-181a and miR-181b mimic transfection is mediated by repression of RASSF1A, we cotransfected miR-181a and miR-181b mimics and a pcDNA3.1/RASSF1A-vector or the corresponding pcDNA3.1/control-vector in NB4 cells. Western blot 48h after transfection revealed a significant repression of cyclin D1 protein when RASSF1A is expressed lacking a 3'UTR in the presence of microRNA mimics (Fig. 7F).

Discussion

A rising number of studies have shown the importance of microRNAs 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,L). Furthermore, we demonstrate that cytostatics and arsenic trioxide which are typically used in APL therapy and predominately inducers of apoptosis does not affect miR-181a/b expression (Figure 1I,J). 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/RAR α and thereby changes gene expression let assume that miR-181a/b expression is dependent on PML/RAR α (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,G,F,H). This observation substantiates the proposed PML/RAR α -dependency of miR-181a/b expression.

The miR-181a/b-cluster has been shown to be upregulated in AML patients with C/EBP α -mutations which have a favorable prognosis and 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 over 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 microRNA cluster as prognostic marker in t(15;17).

Beside its function as transcriptional repressor (2), PML/RAR α 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/RAR α -dependent upregulation of miR-181a/b in PR9 cells and in PML/RAR α knock-in mice (Fig. 2 AC). Additionally, we show significantly higher expression of the miR-181a/b-cluster in bone marrow samples from APL patients (Fig. 2D). These results are reinforced by data from Li et al. (31) and Jongen-Lavrencic et al. (32). Taken together, to the best of our knowledge, we are the first to show PML/RAR α -dependent upregulation of the miR-181a/b-cluster in AML. Since PML/RAR α has no direct binding site in the promoter region of the miR-181a/b-cluster (16) the transcriptional induction has to occur indirect. The exact mechanism how the microRNA 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) let assume that both microRNAs are involved in the formation of the oncogenic phenotype caused by PML/RAR α . Our functional studies show that ectopic expression of miR-181a and miR-181b effectively blocks ATRA-induced granulocytic differentiation (Fig. 3F,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 et al. (33) and Li et al. (34) who assign miR-181a as an antileukemic microRNA 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 et al.,

which showed that miR-181a blocks myeloid differentiation of HL60 and CD34+ hematopoietic stem/progenitor cells (41).

In cancer, oncogenic microRNAs 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 microRNAs which have 3 conserved binding sites in the 3'UTR of RASSF1A (Fig. 4A,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). Since, 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 (Fig. 1A,D,K,L and Fig. 4A). Additionally, 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 could also be shown in hepatocellular cancer stem cells by Meng et al. (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,G,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/RAR α -dependent mechanism is supported by protein expression data in AML patient samples and in PML/RAR α knock-in mice, which show reduced RASSF1A protein levels when the miR-181a/b-cluster is highly expressed (Fig. 2C,D and 5). Additionally, 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 et al. (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 confirm the tumor suppressor function of RASSF1A in APL (Fig. 6C). Additionally, 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 (Figure 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 progress in APL (Fig. 7B, 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,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). Based on these findings, we claim RASSF1A as an important factor in the granulocytic differentiation, which prevents cyclin D1 accumulation, cell cycle progress 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/RAR α -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 microRNAs, transcriptional induced by PML/RAR α , lead to translational repression of the tumor suppressor RASSF1A. Its function is restored through repression of miR-181a/b expression by ATRA-induced degradation of PML/RAR α 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 which seems to be highly specific for APL. Finally, manipulation of miR-181a/b could offer novel treatment strategies in PML/RAR α -associated APL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Précis

Findings identify a pivotal microRNA cluster and tumor suppressor gene as determinants of the outgrowth versus effective therapeutic control of acute promyelocytic leukemias.

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Figure 1. 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 24h after ATRA-treatment of NB4 cells. **C**, **E**, **G**, FACS for CD11b expression in NB4 (**C**), U937 (**E**) and HL60 cells (**G**) 72h after ATRA application. **D**, **F**, **H**, qPCR for miR-181a/b in NB4 (**D**), U937 (**F**) and HL60 cells (**H**) after ATRA application at indicated time points. **I**, **J**, qPCR for miR-181a/b in NB4 cells treated with As₂O₃, doxorubicin w/o cytarabine or ATRA for 24h. **K**, **L**, qPCR for miR-181a/b in bone marrow samples from APL patient at diagnosis

time point (newly diagnosed APL) and while ATRA-treatment (APL during ATRA-based therapy). n.s. signifies not significant. * p-value 0.05; ** p-value 0.01.

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Figure 2. PML/RAR α 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 blot for RAR α and PML/RAR α 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/RAR α 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 AML patients

with indicated subtypes and healthy donors. n.s. signifies not significant. * p-value 0.05;
** p-value 0.01.

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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. The diagram represent the amount of Annexin V⁺ / 7-AAD⁻ cells in each condition. **C**, Replating assay of miR-ZIP-181a/b or miR-ZIP-control expressing NB4 cells. Pictures represent one well of a 12 well plate as an example of each condition at the first and the third plating. **D**, Colony size measurement of NB4 cells stably

expressing miR-ZIP-181a/b or miR-ZIP-control sequences. Pictures represent one example for each condition. Bars in the right corner of the picture corresponds to 300µm. **E**, Cell growth curve of miR-ZIP-181a/b or miR-ZIP-control expressing NB4 cells. **F**, FACS for ATRA-induced CD11b expression 48h 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 48h after transfection. * p-value 0.05; ** p-value 0.01.

Figure 4. The tumor suppressor RASSF1A is a direct target of the miR-181a/b-cluster
A, Western blot for RASSF1A protein in ATRA and DMSO treatment of NB4 cells at indicated time points. **B**, Western blot for RASSF1A protein in As₂O₃ or with the control treated NB4 cells at indicated time points. **C**, Schematic representation of the RASSF1A 3'UTR including the three predicted miR-181 binding sites. **D**, Schematic representation of the pGL3-constructs for the wild type 3'UTR of RASSF1A and the mutated 3'UTR of RASSF1A. **E**, Schematic representation of the predicted and mutated miR-181 binding sites in the RASSF1A 3'UTR. Numbers behind the sequence represent the position of the

nucleotides relative to the termination codon of human RASSF1A. **F**, Luciferase assay was performed in U937 cells 24h after cotransfection of pGL3-3'UTR RASSF1A (wild type or mutated) or pGL3-control vector and miR-181a, miR-181b or control mimics. Bars represent the luciferase activity for the corresponding vectors. Normalization was done by Renilla luciferase. **G**, Western blot for RASSF1A protein 48h after miR-181a, miR-181b or control (nc) mimic transfection in U937 cells. **H**, Western blot for RASSF1A protein in NB4 cells stably expressing miR-ZIP-181a, miR-ZIP-181b or miR-ZIP-control sequences. * p-value < 0.05.

Figure 5. RASSF1A protein is specifically suppressed by PML/RAR α

A, Western blot for RASSF1A protein in bone marrow samples of patients with different AML subtypes and cell samples from non-leukemic 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 blot for RASSF1A protein in bone marrow samples from PML/RAR α

knock-in mice (C57BI/6-mCG^{+/PR}) and wild type mice (C57BI/6-WT), (right). The number of mice (n) or patients (n) in each group is shown below the bars. * p-value 0.05.

Figure 6. RASSF1A is essential for ATRA induced granulocytic differentiation and induces apoptosis in APL

A, qPCR for RASSF1A mRNA in NB4 cells stably expressing RASSF1A shRNA sequences or an unspecific control. **B**, FACS for CD11b expression 48h and GCSF-R expression 72h after ATRA application. The diagrams next to the histograms show the amount of CD11b or GCSF-R positive cells (%) for each condition. **C**, Annexin V (PE)/7-AAD staining of NB4 cells 24h after overexpression of RASSF1A. The diagram shows the Annexin V+/ 7AAD- fraction of pcDNA3.1-RASSF1A and pcDNA3.1-nc (control vector) transfected cells. * p-value 0.05; ** p-value 0.01.

Figure 7. MiR-181a/b and RASSF1A modulate differentiation and apoptosis in APL via regulation of cyclin D1

A and **B**, Western blot for cyclin D1 protein in NB4 cells 48h after ATRA application (**A**) and 24h after overexpression of pcDNA3.1-RASSF1A or pcDNA3.1-nc (**B**). **C**, Cell cycle of NB4 cells stably expressing RASSF1A specific shRNA sequences 24h after ATRA application. **D**, Cell cycle of miR-ZIP-181a, miR-ZIP-181b or miR-ZIP-control expressing NB4 cells. **E**, Western blot for cyclin D1 in U937 cells 48h after miR-181a, miR-181b or control mimic transfection. **F**, Western blot for cyclin D1 protein 48h after cotransfection of miR-181a, miR-181b or control mimics and pcDNA3.1-RASSF1A or the pcDNA3.1-nc-

vector. **G**, Schematic representation of ATRA and PML/RAR α -dependent regulation of the miR-181a/b-cluster and RASSF1A. The oncogenic fusion protein PML/RAR α leads to the transcriptional induction of miR-181a and miR-181b. Both microRNAs block RASSF1A protein synthesis which leads to cell cycle progression and proliferation via accumulation of cyclin D1. Treatment of APL blasts with pharmacological doses of all-*trans*-retinoic acid (ATRA) leads to the destruction of the PML/RAR α fusion protein followed by repression of miR-181a/b transcription *in vitro* and *in vivo*. This results in the recovery of RASSF1A protein synthesis which leads to cell cycle arrest and differentiation as well as apoptosis through inhibition of cyclin D1 accumulation. n.s. signifies not significant. * p-value 0.05; ** p-value 0.01.