Transcriptional repression of microRNA genes by PML-RARA increases expression of key cancer proteins in Acute Promyelocytic Leukemia

Anne Saumet1, #, Guillaume Vetter2, Manuella Bouttier3, Elodie Portales-Casamar4, Wyeth W. Wasserman4, Thomas Maurin5, Bernard Mari5, Pascal Barbry5, Laurent Vallar6, Evelyne Friederich5, Khalil Arar7, Bruno Cassinat8, Christine Chomienne8 and Charles-Henri Lecellier3, *

1Institut de Génétique Humaine, CNRS UPR1142, F-34396 Montpellier cedex 5, France
2Université du Luxembourg, L-1511 Luxembourg, 3Institut de Génétique Moléculaire de Montpellier, CNRS UMR5535-IFR122, Université de Montpellier, F-34293 Montpellier cedex 5, France 4University of British Columbia, BC V5Z 4H4 Vancouver, Canada, 5Institut de Pharmacologie Moléculaire et Cellulaire, UMR6097 CNRS/UNSA, F-06560 Sophia Antipolis, France, 6CRP-Santé, Luxembourg, L-1511 Luxembourg, 7Sigma-Proligo, F-91030 Evry cedex, France, 8Institut Universitaire d’Hématologie, INSERM U718, Hôpital St Louis, 75475 Paris cedex 10, France.

# present address: IRCM, Institut de Recherche en Cancérologie de Montpellier- INSERM, U896 - Université Montpellier1 - CRLC Val d’Aurelle Paul Lamarque, Montpellier, F-34298, France

* Correspondence
Charles-Henri Lecellier
Institut de Génétique Moléculaire de Montpellier - CNRS UMR 5535 - IFR 122
Université de Montpellier
1919 Route de Mende – F-34293 Montpellier Cedex 5
Phone : 33 (0) 4 67 61 36 64
Fax : 33 (0) 4 67 04 02 31
E-mail : charles.lecellier@igmm.cnrs.fr

Category: Neoplasia

Copyright © 2008 American Society of Hematology
Abstract

Micro(mi)RNAs are small non-coding RNAs that orchestrate many key aspects of cell physiology and their deregulation are often linked to distinct diseases including cancer. Here, we studied the contribution of miRNAs in a well characterized human myeloid leukemia, Acute Promyelocytic Leukemia (APL), targeted by retinoic acid and trioxide arsenic therapy. We identified several miRNAs transcriptionally repressed by the APL-associated PML-RAR oncogene which are released after treatment with all-trans retinoic acid. These co-regulated miRNAs were found to control, in a coordinated manner, crucial pathways linked to leukemogenesis, such as HOX proteins and cell adhesion molecules whose expressions are thereby repressed by the chemotherapy. Thus, APL appears linked to transcriptional perturbation of miRNA genes and clinical protocols able to successfully eradicate cancer cells may do so by restoring miRNA expression. The identification of abnormal miRNA biogenesis in cancer may therefore provide novel biomarkers and therapeutic targets in myeloid leukemias.
Introduction

The micro(mi)RNAs are ~22nt-long RNAs that orchestrate the expression of genes involved in many aspects of cell biology (reviewed in \(^1,^2\)). The human genome encodes more than 500 miRNAs located in introns, exons or intergenic regions and it is now admitted that each cell type produces a specific miRNA repertoire. Most miRNA genes are transcribed in a manner similar to coding genes but differ in their mode of post-transcriptional processing \(^1\). As a consequence of the fundamental functions of miRNAs, their deregulations are implicated in diverse human pathologies, in particular cancers (reviewed in \(^3\)). MiRNAs are now considered as oncogenes or tumor suppressors and represent promising diagnostic and prognostic markers \(^3\). MiRNAs are also envisaged as novel targets of therapeutic strategies \(^4\). The identification of miRNAs implicated in cancer might therefore not only help getting a better understanding of the molecular basis of human diseases, but also characterize novel biomarkers and therapeutic targets of cancers. Within this context, we studied the potential contribution of miRNAs in Acute Promyelocytic Leukemia (APL). APL is identified as the M3 subtype of acute myeloid leukemia (AML) by the French-American-British classification and is characterized by a differentiation arrest of granulopoiesis at the promyelocytic stage (\(^5\) and references herein). APL is associated with chromosomal translocations that invariably implicate the gene encoding the retinoic acid receptor alpha (RARA). The resulting fusion proteins exert dominant and negative effect on RARA and hence, retinoic acid (RA)-regulated genes become insensitive to physiological doses of RA. The most frequent translocation fuses the RARA with the promyelocytic leukemia protein (PML) gene \(^6\). APL is considered as the most malignant form of acute leukemia with a severe bleeding tendency and a fatal course of only weeks. However, pharmacological doses of different RAR agonists, such as all-trans-retinoic acid (ATRA), overcome the PML-RARA-mediated repression and restore normal
transcription and granulocytic differentiation. Providing our knowledge of APL leukemogenesis and therapy, this disease is considered as an excellent model of cancer therapies. APL therefore appeared appropriate to study the exact contribution of miRNAs in the initiating events of leukemia and in associated chemotherapies.

In this study, using bioinformatics and transcriptomics approaches followed by experimental validations, we identified a group of miRNAs targeted by PML-RARA. We further show that the repression exerted by PML-RARA/RXR complexes can be stopped by ATRA even in primary blast cells. Some of the identified miRNAs were also shown to be regulated by RARA in non-APL cells. Finally, transcriptomics analyses of mRNAs modulated by ATRA in distinct APL cell lines allowed identifying targeted messengers which are implicated in crucial pathways linked to leukemogenesis, such as HOX proteins and cell adhesion molecules (notably the Urokinase Receptor, uPAR).
Material and Methods

Cells and treatment. The NB4, NB4-LR1 and NB4-LR2 cells were cultured in RPMI (Invitrogen) as previously described \(^7\) and treated with 1\(\mu\)M ATRA (Sigma). 293T cells were maintained in DMEM (Invitrogen) supplemented with 2mM L-glutamine, 100 \(\mu\)g/ml penicillin, 50 \(\mu\)g/ml streptomycin and 10% heat-inactivated fetal calf serum. Primary blast cells extracted from the bone marrow of APL patients were treated for 6 days with ATRA (0.1\(\mu\)M). The diagnosis of APL was established according to clinical presentation and morphological criteria of the FAB classification and was subsequently confirmed by cytogenetic assays and RT-PCR analyses for detection of the t(15;17) translocation and PML-RARA transcripts. All patients had consented to the use of their medical records. The study was approved by the Ethics Committee of participating institutions and informed consent was obtained in accordance with the Declaration of Helsinki.

miRNA microarrays. Small RNA fraction was purified with the miRvana miRNA isolation kit (Ambion), labeled with Alexa fluor dyes and hybridized in a dye-swap experiment (Agilent) on specific chips (http://www.microarray.fr/microRNA). TIF images containing the data from each fluorescence channel were quantified with the Genepix pro 6.0 program (Axon Instruments) using a ‘circular features’ quantification method. Normalizations were performed using the limma package available from Bioconductor (http://www.bioconductor.org). Intra slide and inter slide normalization were performed using the Print Tip Loess and the quantile methods respectively and means of ratios from ATRA treated/control were calculated for each cell line. Experimental data and associated microarray designs have been deposited in the NCBI Gene Expression Omnibus (#GSE11379).
cDNA microarrays. Total RNA samples were amplified using the Amino Allyl MessageAmp II aRNA Amplification kit (Ambion) and coupled to Cy3 or Cy5 dyes (Amersham Biosciences). Whole human genome arrays (Human Operon version 2, University Medical Center of Utrecht) were used. For each condition, 3 independent hybridizations including one dye-swap hybridization were realized. For statistical analyses using the SAM method, the two classes unpaired response type was used. Potentially interesting genes resulting of the SAM analysis were selected at a False Discovery Rate of 5%.

DNA contracts and Luciferase Assays. The miR-23a/24-2 promoter was cloned into the BglII/HindIII restriction sites of pGL3b vector (Promega). All sets of primers are indicated in Supplemental table S1. The predicted PML-RARA response element was mutagenized using the QuickChange Mutagenesis procedure (Stratagene). The 3′UTRs of uPAR and EBF-3 mRNAs were fused to the renilla gene using the XhoI/NotI restrictions sites of the psiCHECK2 vector (Promega). The pcDNA3 vector encoding PML-RARA is described in 9. Transfections of 293T cells were performed using Lipofectamine 2000 (Invitrogen). When indicated, ATRA treatment (1μM, 16 hours) was realized 24 hours after transfection. Luciferase assays were performed using Dual-Luciferase (Promega). The pRLTK vector was used to normalize the experiments except for those conducted with psiCHECK2 which contains a firefly gene internal control. LNAs (supplemental table S1) were provided by Sigma-Proligo. Indicated results are means of at least three independent experiments.

Quantitative RT-PCR. Total RNAs were extracted using Trizol (Invitrogen) for NB4, HL-60 and 293T cell lines or RNAplus (QBiogen) for primary blast cells. RTs were realized using oligodT(N) for RARB, uPAR, HOXB8 and GAPDH or specific stem-loop oligonucleotides indicated in supplemental table S1 for miRNAs. The miRNA-specific RT-qPCR protocol was
adapted from \(^{10}\) to SYBR Green PCR (Roche). Indicated results are means of at least three independent experiments.

**Chromatin Immunoprecipitation.** ChIP experiments were performed using a standard protocol. Briefly, the cross-linked chromatin was sonicated and immunoprecipitated overnight at 4°C by using antibodies against PML, RARA or RXRA (10mg) (Santa-Cruz). Immunoprecipitated DNA was used as template for PCR and qPCR using sets of primers indicated in supplemental table S1.
Results

Prediction of PML-RARA response elements in miRNA genes
To identify potential transcription factor binding sites (TFBS) for the PML-RARA fusion protein, microRNA regulatory regions were analyzed with the NHR-Scan system\textsuperscript{11}. The most common TFBS prediction methods, position weight matrices, are inappropriate for the analysis of nuclear receptor target sites due to the tolerance of these transcription factors for paired half-sites with variable spacing and orientation. NHR-Scan uses a flexible Hidden Markov Model (HMM) framework to capture those characteristics. We constructed a specific HMM model for the PML-RARA fusion protein using the results from a selection/amplification experiment\textsuperscript{12}. The PML-RARA-specified form of NHR-Scan was used to predict binding sites in the promoter regions of 247 human intergenic microRNAs (from a miRNA promoter dataset provided by Mahony\textit{et al.}\textsuperscript{13}). We limited the search space to the 2kb upstream of the transcription start site (TSS) or, in the event of a proximal upstream gene, to the intergenic region. For adjacent microRNAs separated by less than 250 bp, 5'-most TSS was considered. As shown in Table 1, 65 out of 247 microRNA promoters contain a PML-RARA predicted site.

Transcriptomics analyses of the miRNA modulations induced by ATRA in NB4 cells
Next, we reasoned that if some miRNAs are repressed by PML-RARA, then pharmacological doses of RA should abolish this repression and lead to an increase in the level of expression of the corresponding miRNAs. This expression pattern should in fact be similar to those observed for known RA-regulated genes, such as RARB (or RARb2), a well characterized target of the RARA and PML-RARA proteins\textsuperscript{14}. We treated NB4 cells\textsuperscript{7} with ATRA 1μM for 16 hours as this concentration induces NB4 maturation in 4 days (Fig. 1A) and this time
point was previously shown to be appropriate to observe gene expression fluctuations. First, using RT-qPCR, we confirmed that ATRA induced an up-regulation of the RARB mRNA (Fig. 1B), indicating that our settings were suitable to detect transcriptional modulations induced by ATRA. The same RNA samples were then analyzed using miRNA microarrays (Fig. 1C). These analyses revealed that ATRA readily modulated several miRNAs among those the miR-181b, which was previously shown to be down-regulated by ATRA in NB4 cells, the miR-15b, the miR-223 and the miR-342, 3 miRNAs previously found up-regulated in similar settings (Fig. 1C). The miR-106a, miR-129-1, miR-20b were also found down-regulated by ATRA while the miR-143, miR-30c and miR-378 were up-regulated (Fig. 1C). Only 2 miRNAs, among the 65 bioinformatically predicted to be repressed by PML-RARA, were detected by the arrays: the miR-210 and the miR-513-2 (compare Fig. 1C with Table 1). While the miR-210 was clearly up-regulated by ATRA, suggesting that this miRNA could be an authentic transcriptional target of PML-RARA, the miR-513-2 was down-regulated excluding this miRNA as a candidate.

RT-qPCR analyses of the miRNA modulations induced by ATRA in NB4 cells

In order to identify additional miRNA candidates, we performed miRNA-specific RT-qPCRs directed against 11 out of the 65 miRNAs presented in Table 1 (Fig. 1D). The miR-223, the miR-181a and the miR-181b, detected by microarrays, were also amplified (Fig. 1D). Whereas RT-qPCRs confirmed the results obtained by microarrays for the miR-223, miR-181a, miR-181b and miR-210, this approach revealed that the miR-10b, miR-194, miR-195, miR-196a, miR-23a, miR-377 and miR-622 were also up-regulated by ATRA in NB4 cells (Fig. 1D). Some miRNAs (miR-331-5p) could not be detected and the expression of others (miR-133b and miR-146a) did not follow the expected pattern of PML-RARA-repressed genes (Fig. 1D). Overall, the RT-qPCR analyses corroborated 75% of our predictions and
suggested that the miR-10b, miR-194, miR-195, miR-196a, miR-210, miR-23a, miR-377 and miR-622 could represent authentic targets of PML-RARA. To validate our findings, investigations were further focused on the miR-210 and the miR23a (belonging to a cluster of miRNAs, namely miR-23a, miR-27a and miR-24-2). Of note, since the miR-23a, miR-27a and miR-24-2 are generated from the same primary miRNA and because clustered miRNAs exhibit similar expression pattern, we considered that the miR-23a was representative of the expression of the entire miRNA cluster.

**Transcriptional repression of the miR-210 and the miR23a/24-2 by PML-RARA**

To directly confirm the binding of PML-RARA to the miR-210 and miR-23a/24-2 promoters in NB4 cells, we performed chromatin immunoprecipitation (ChIP) experiments using anti-PML, anti-RARA and anti-RXRA antibodies. Anti-RXRA immunoprecipitations were performed because, although PML-RARA is able to bind DNA in the absence of RXR, the fusion protein has a greater DNA-binding affinity when complexed with RXRA. The RARB promoter was used as a positive control while the miR-223 promoter was used as a negative control (Table 1). Whereas the miR-223 promoters were not associated with PML, RARA or RXRA, these three proteins were physically bound to the RARB as well as the miR-210 and the miR-23a/24-2 promoters (Fig. 2A and B), suggesting that these miRNAs are repressed by PML-RARA/RXRA complexes in APL cells, similarly to the RARB promoter. As RXRA was recently shown to be an essential factor in APL pathogenesis, these findings might draw attention to the importance of the transcriptional repression of miRNAs in the development of APL. The sequence located directly upstream the miRNA precursor does not necessarily define the miRNA promoter and the formal characterization of the transcriptional start site of the primary miRNA is required before cloning miRNA promoters. We took advantage of the study performed by Lee et al. and cloned the miR-
23a/24-2 promoter to drive the expression of the firefly luciferase reporter gene. The reporter was transfected in 293T cells together with a PML-RARA expression vector \(^9\). We observed that PML-RARA readily reduced the transcriptional activity of the miR-23a/24-2 promoters even in non APL cells (Fig. 2C). This reduction was limited by the addition of 1μM ATRA (Fig. 2C). In accordance with our bioinformatics predictions, a mutant of the miR-23a/24-2 promoter corresponding to the predicted PML-RARA response element was not sensitive to PML-RARA expression (Fig. 2C). These results indicated that PML-RARA repressed the miR-23a/24-2 promoter through the response element identified \(\text{in silico}\). Furthermore, RA did not induce noticeable changes in the expression of the miR-23a and the miR-210 in PML-RARA-lacking HL-60 cells treated with ATRA for 16 hours (Fig. 3A) \(^22\). On the other hand, degrading PML-RARA with arsenic trioxide in NB4 cells increased the expression of the miR-23a, the miR-210 and the RARB (Fig. 3B) \(^23\). These results confirmed that the expression of the miR-23a and the miR-210 is dependent on PML-RARA.

**Evaluation of the miRNA modulations induced by ATRA in primary APL blast cells**

Next, we determined whether the miRNA modulations observed in NB4 cell line were biologically relevant and tested miRNA expression levels in primary APL blast cells extracted from the bone marrow of 3 different patients. RT-PCR analyses performed during diagnosis revealed that patient #2 expressed the short BCR3 isoform of PML-RARA (11,000 copies) while patients #1 and #3 expressed the long BCR1 isoform (33,900 and 18,900 copies respectively). The blast cells were treated with 100 nM of ATRA for 6 days and RNA extraction was performed each day from day 0 to day 4 of treatment \(^24,25\). Cell viability and maturation of APL cells was assessed by Trypan Blue (Fig. 4A) and NitroBlueTetrazolium (NBT)-dye reduction assays (Fig. 4B) respectively at day 0, 3 and 6 of ATRA treatment. We also controlled the induction of the prototypic RARB by ATRA (Fig. 4C). RT-qPCRs specific
for the miR-23a, miR-210, miR-223 and miR-181a were then performed (Fig. 4D, E, F and G, respectively). The miR-181a was chosen because the expression of this miRNA was recently shown to correlate with morphological sub-class of AML. We observed that the expression of the miR-23a, miR-210 and miR-223 increased throughout the ATRA treatment although the fold changes were not exactly comparable, the miR-223 being the least induced miRNA (Fig. 4D, E and F). The expression of the miR-181a was generally diminished by ATRA (Fig. 4G). Hence, the modulations of the miR-23a, miR-210, miR-223 and miR-181a induced by ATRA in NB4 cell line were confirmed in primary blast cells.

Transcriptional regulation of the miR-210 and the miR23a/24-2 by RARA in non APL cells

PML-RARA is thought to interfere with the functions of both parental proteins in a dominant negative manner. However, although PML-RARA binds to canonical RARA binding sites, it also recognizes wider range of DNA-target sequences that are not regulated by RARA. Therefore, we tested whether the miR-210 and the miR-23a/24-2 were also regulated by RARA in non APL cells. The promoters of the miR-210 and miR-23a/24-2 exhibited response elements compatible with the binding of RA-integrating complexes (i.e. RARA/RXRA heterodimers) (Table 1). We verified, using RT-qPCRs, the effect of physiological doses (10 nM) of RA on the expression of the miR-210 and the miR-23a/24-2 in non APL 293T cells. As shown in Figure 5A, the miR-210 and miR-23a were rapidly up-regulated by physiological doses of ATRA while no modulation of the miR-223 expression could be detected in the same time frame. These observations suggested that the miR-210 and the miR-23a/24-2 could be transcriptionally modulated by RA in non APL cells. ChIP experiments directed against both RARA and RXRA in 293T cells revealed that RARA/RXRA heterodimers could bind the miR-210 as well as the miR-23a/24-2 promoters but not the miR-
Luciferase assays were also conducted to substantiate these regulations. We transfected the firefly reporter containing the miR-23a/24-2 promoter in 293T cells and treated the cells with increasing doses of ATRA (from physiological – 10 nM - to pharmacological doses - 1μM) for 16 hours (Fig. 5D). We observed that 10 nM ATRA was sufficient to induce a significant increase in the luciferase activity (Fig. 5D) indicating that the miR-23a/24-2 promoter was sensitive to physiological doses of ATRA. Moreover, we also tested the mutant described in the Figure 2C and observed that this mutant was not responsive to ATRA 10 nM (Fig. 5E). This result showed that, in the case of the miR-23a/24-2 promoter, the PML-RARA response element is similar to the RARA response element. Together, these results revealed that the miR-210 and the miR-23a/24-2 are directly repressed by the PML-RARA oncogene in APL cells and are regulated by RARA/RXRA heterodimers in non APL cells.

Functional consequences of the PML-RARA-mediated miRNA repression

Next, we evaluated the functional consequences of PML-RARA-mediated miRNA repression in NB4 cells and used transcriptomics approaches to identify mRNAs potentially regulated by the group of miRNAs repressed by PML-RARA. In fact, simultaneous profiling of miRNA and mRNA expression is an appropriate strategy to identify functional miRNA targets. We reasoned that the up-regulation of miRNAs induced by ATRA should be accompanied by the down-regulation of the corresponding mRNA targets. We analyzed the RNA samples used in Figure 1 using pan-genomic cDNA microarrays. In order to focus only on genes that are both sensitive to ATRA and PML-RARA repression (and, hence, possibly to PML-RARA-repressed miRNAs), we compared these results to those obtained with RNA samples extracted from ATRA-resistant APL cells, NB4-LR1 and NB4-LR2 (Supplemental table S3). The NB4-LR1 cells do transcriptionally respond to ATRA but do not maturate. In contrast, the
NB4-LR2 cells show a clear defect in RA signalling\(^9\). We reasoned that, in contrast to genes modulated by other pathways, RA-sensitive genes should be up-regulated by ATRA in both NB4 and NB4-LR1 cells but remain unchanged in NB4-LR2 cells. This expression pattern was indeed observed in the case of the PML-RARA-targeted RARB gene (Fig. 1B and Supplemental figure S1). Of note, the increase of the RARB mRNA was less pronounced in NB4-LR1 than in NB4 cells, likely reflecting the activation of additional pathways in NB4 cells, such as the PKA pathway, that might synergize with ATRA\(^{31}\). Genes that were found up- or down-regulated in NB4 and NB4-LR1 cells but unchanged in NB4-LR2 are recapitulated in supplemental tables S2 and S3 respectively. We noticed that global changes of the transcriptome were less pronounced in NB4-LR1 than in NB4 cells, again likely reflecting the activation of other cellular pathways by ATRA in NB4 cells\(^ {31}\). Overall, our microarray analyses were consistent with those previously published\(^ {25,32}\). The lists of the up- and down-regulated genes were then compared to the list of miRNAs bioinformatically predicted to be repressed by PML-RARA using the miRBase Targets (http://microrna.sanger.ac.uk/targets/v5/). Among the first hundred genes analyzed in each case, we observed that 77% of the down-regulated genes and 79% of the up-regulated genes could potentially be targeted by at least one miRNA predicted to be repressed by PML-RARA (Fig. 6A). Hence, no significant enrichment in down-regulated genes could be observed. However, translational repression orchestrated by miRNAs generally requires several targets of the same miRNA and/or distinct targeting miRNAs\(^2\). As our studies identified a subset of miRNAs that are coordinately induced by ATRA, it is likely that these miRNAs do not exert their action independently but rather synergize and target the same mRNAs. Thus, we counted the number of miRNAs potentially repressed by PML-RARA targeting each mRNA indicated in supplemental tables S2 and S3 (Fig. 6A). These analyses revealed that 9 out of the 100 down-regulated mRNAs could be targeted by more than 9 PML-RARA-repressed miRNA
candidates as opposed to only 1 out of the 100 up-regulated mRNAs. In order to confirm that these genes were genuine miRNA targets, we considered the 4 down-regulated mRNAs targeted by 10 or more miRNA candidates (Fig. 6B). While only few data were available for three of those, the uPAR gene was particularly appealing because this gene has already been studied in the context of AML, NB4 and ATRA response.

MiRNAs repressed by PML-RARA control key cancer genes

The uPAR protein plays essential roles in a variety of cell functions that exploit extracellular proteolysis, adhesion and chemotaxis (reviewed in). The uPAR expression level has been strongly correlated with poor prognosis in a variety of malignant tumors and patients suffering with AML have high expression of uPAR and high relapse risk after therapy. In addition, the uPAR mRNA was specifically shown to be down-regulated by ATRA in NB4. We first validated microarray analyses by RT-qPCRs and confirmed that the uPAR mRNA was down-regulated by ATRA in NB4 and NB4-LR1 but not in NB4-LR2 cells (Fig. 6C). The miRBase Targets revealed that the 3’UTR of uPAR harbored 13 sequences that might be targeted by several miRNAs predicted to be repressed by PML-RARA (Fig. 6D). To test these predictions, we cloned the 3’UTR of uPAR downstream the renilla luciferase reporter gene (psiCHECK2-uPAR 3’UTR) and transfected this construct into 293T cells (Fig. 7A). We observed that the renilla expression was drastically reduced when fused to uPAR 3’UTR (Fig. 7A). We additionally observed that the introduction of PML-RARA in 293T cells restored the expression of the renilla reporter containing the uPAR 3’UTR whereas the fusion protein had little effect on the expression of the parental renilla (Fig. 7A). Moreover, treatment of the transfected cells with 1μM ATRA abolished the effect of PML-RARA on psiCHECK2-uPAR 3’UTR (Fig. 7A). In parallel, we verified that PML-RARA repressed the expression of the RARB mRNA whereas the addition of ATRA 1μM stopped this repression.
(Fig. 7B). A similar pattern was obtained for the miR-195 (Fig. 7B), one of the 11 miRNAs potentially targeting uPAR mRNA and suspected to be repressed by PML-RARA (Fig. 6D and Fig. 1D). It is noteworthy that, in both cases, the addition of ATRA 1μM slightly increased the RNA expression (Fig. 7B), suggesting that the miR-195, similarly to the RARB, is sensitive to RA. These observations indicate that the modulation exerted by the uPAR 3’UTR is inversely correlated to the capacity of PML-RARA to repress transcription. We also tested a candidate gene targeted by less than 11 miRNAs and evaluated the effect of the 3’UTR of the EBF-3 mRNA, targeted by only 2 miRNAs out of the 65 indicated in Table 1 (Supplemental table S3 and Fig. 7C). While the 3’UTR of EBF-3 clearly decreased renilla expression, potentially due to negative post-transcriptional regulations, this effect was insensitive to PML-RARA expression (Fig. 7C). These results suggested that EBF-3 is not a target of the miRNAs repressed by PML-RARA and underscored the importance of the number of miRNA binding sites per target. Next, we verified that the regulation elicited by the 3’UTR of uPAR was orchestrated by miRNAs. For that purpose, we transfected 293T cells with the psiCHECK2-uPAR 3’UTR vector and specific LNA miRNA inhibitors (Fig. 7D). A functional LNA anti-miR-32 was used as a negative control. The inhibition of the miRNAs let-7a, let-7c, let-7d, miR-133b, miR-146a had a modest effect on the expression of the reporter harboring the 3’UTR of uPAR whereas the inhibition of the miR-194, miR-195, miR-331-5p, miR-331-3p, miR-377 and miR-622 significantly restored the expression of the Renilla (Fig. 7D). Mixing LNAs directed against the miR-133b and the miR-195 had a stronger effect than each LNAs transfected alone (Fig. 7D). Likewise, the strongest effect on Renilla expression was observed when all LNAs were mixed (Fig. 7D). This might suggest that this subset of miRNAs act in a synergistic manner. The similar effects of the LNAs directed against the miR-377 and the miR-622 might also indicate that these miRNAs act in a redundant manner. Overall, these observations showed that the miRNAs repressed by PML-
RARA do not exert their action independently but are rather coordinated to regulate the same mRNAs. Finally, some of these miRNAs (i.e. miR-331-5p) could not be detected in NB4 cells but seemed efficient in regulating uPAR 3’UTR in 293T cells. This indicated that distinct miRNAs could regulate uPAR expression depending on the cell type, a proposal consistent with the idea that each cell type harbors a particular miRNA repertoire.

Finally, we also inspected validated miRNA targets whose expression was shown to correlate with some miRNAs of our subset. Notably, our bioinformatics and RT-qPCR analyses have revealed that the miR-196a might be repressed by PML-RARA (Table 1 and Fig. 1D). This miRNA is known to regulate the expression of the HOXB8 mRNA. HOXB8 is also predicted to be repressed by the miR-27a, miR-377, miR-520d and miR-524, which are validated or potential targets of PML-RARA (Table 1 and Fig. 2). Using specific RT-qPCR, we found that the level of this messenger was down-regulated by ATRA in NB4 and NB4-LR1 while remaining unchanged in NB4-LR2 (Supplemental figure S5). We also found using specific RT-qPCR that the level of HoxB8 messenger was systematically increased in 293T cells when blocking the miR-377 (Supplemental figure S5). This results suggested that HOXB8 expression could be coordinately regulated by several miRNAs, in particular the miR-377 and the miR-196a.
Discussion

We revealed that PML-RARA is able to transcriptionally repress several miRNA genes. Because the expression of these miRNAs is restored by ATRA and As2O3, our results suggest that clinical protocols, able to successfully eradicate cancer cells, may do so at least in part by impacting miRNA expression. We also showed that the miR-23a/24-2 and miR-210 are physiologically regulated by RA. These findings indicate that, in addition to its canonical properties of transcription regulation, RA, through miRNAs, can indirectly affect post-transcriptional processes such as translation.

Our miRNA microarray analyses and those performed by Garzon et al. 16 converged to the findings that the miR-15b, miR-223 and miR-342 are up-regulated by ATRA whereas the miR-181a and miR-181b are down-regulated in NB4 cell lines. The diminution of the expression of miR-181a induced by ATRA was confirmed in primary APL blasts (Fig. 4E). The expression of this miRNA was recently correlated with a particular subclass of AML as the miR-181a is highly expressed in AML-M1 or AML-M2 compared to AML-M4 or AML-M5 26. No AML-M3 sample was tested in this study 26. As the miR-181a is down-regulated by ATRA (Fig. 1D and 3E), our observations might suggest that the expression of the miR-181a is also relevant in the case of AML-M3. The up-regulation of the miR-223 in ATRA-treated NB4 cells was also observed by Fazi et al. and was implicated in NB4 cell maturation 17. The miR-223 has also been implicated in AML-M2 leukemia wherein it is transcriptionally silenced by the AML1/ETO oncogene associated with the t(8;21) translocation 38. Interestingly, the let-7a, let-7c and let-7d miRNAs were found up-regulated by ATRA in APL cells 16 and we predicted those miRNAs to be repressed by PML-RARA (Table 1). We decided not to include these miRNAs in our RT-qPCR analyses because we suspected that
their high degree of homology could make difficult their discrimination by PCR. However, given the essential functions of the let-7 targets \(^{16,39}\), it may be interesting to further test whether the let-7a, let-7c and let-7d are repressed by PML-RARA.

The findings that PML-RARA represses miRNA genes, including miRNAs located in intergenic regions, reveal that the fusion protein affects unsuspected regions of the chromatin. Our results in fact extend previous findings obtained by Hoemme et al. who showed, using ChIP to chip approach, that PML-RARA regulates key cancer coding genes \(^{40}\). The miRNAs repressed by PML-RARA are also implicated in the control of crucial pathways linked to leukemogenesis. For instance, the expression of HOXB8 seemed to be controlled by miRNAs potentially repressed by PML-RARA (Fig. 1 and Fig. 8). RA is known to induce a temporal program of HOX gene expression and this expression is often perturbed in leukemias \(^{25,41,42,43,44}\). Of note, the miR-10b, which is potentially repressed by PML-RARA (Table 1 and Fig. 1D), also regulates HOXD10 \(^{45}\). The up-regulation of HOX genes in AML due to the down-regulation of miRNAs has already been suspected \(^{26,46,47}\) but we revealed here a potential link with oncogene-mediated transcriptional repression at least in the case of APL. Likewise, we identified the uPAR-coding mRNA as a target of miRNAs repressed by PML-RARA. High expression of uPAR reflects a significant lower remission rate after chemotherapy and a higher risk for relapse \(^{34}\). Curiously, Mustjoki et al. have shown that 13-trans RA increased uPAR mRNA and protein levels in NB4 cells \(^{48}\). However, the authors showed that the global effect of RA was a decrease in proteolytic activity due to the activation of plasminogen activator inhibitors (PAI) \(^{48,49}\). Although we and others have found that ATRA rather decreases uPAR mRNA in NB4 (Fig. 6C and \(^{32,35}\)), our results are consistent with the observation that the overall outcome of ATRA is a decrease in uPA activity \(^{49}\). But, in addition to PAI \(^{49}\), we showed that the miRNA pathway could also be implicated in this
process. This regulation appeared orchestrated by several miRNAs organized in a complex but synchronized network. Although further investigations are required to evaluate the extent of this type of process, our results suggested that coordination may play central role(s) in the action of co-regulated miRNAs.
Acknowledgments

We are grateful to the patients for their contribution. We thank Michel Lanotte for cells and reagents. We are grateful to Valérie Courgnaud, François Bernardin and Mikalail Yatskou for technical advices and help. This work was supported by INSERM, CNRS and the National Research Fund of Luxembourg.

Authorship and Conflict of Interest Statement

References

44. Knoepfler PS, Sykes DB, Pasillas M, Kamps MP. HoxB8 requires its Pbx-interaction motif to block differentiation of primary myeloid progenitors and of most cell line models of myeloid differentiation. Oncogene. 2001;20:5440-5448.
Figure Legends

Table 1. List of microRNAs carrying a predicted PML-RARA binding site in their promoter. The site type describes the half-site combination (IR=Inverted Repeat, DR=Direct Repeat, ER=Everted Repeat) followed by the spacer size (eg. DR2 refers to a direct repeat with 2 bp between the half-sites).

Figure 1. miRNA profiles of NB4 cell line upon ATRA-treatment. A. Morphology of May-Grünwald-Giemsa(MGG)-stained NB4 cells treated or not with ATRA (1µM) for 4 days. Maturation was monitored by the Nitroblue Tetrazolium (NBT) dye reduction assay and the percentage of NBT positive cells obtained at day 4 of treatment is indicated (bottom left). B.C.D NB4 cells were treated with 1 µM ATRA for 16 hours. B. RT-qPCR analysis of RARB mRNA expression in NB4 cells upon ATRA treatment. Results are indicated as fold change, 1 being the cycle threshold (Ct) obtained in the absence of treatment. C. Table of miRNAs modulated by ATRA. See the Experimental Procedures section for details. D. miRNA-specific RT-qPCRs performed in untreated (black histograms) and ATRA-treated NB4 cells (grey histograms). The asterisk (*) indicates an absence of amplification. Results are indicated as fold change, 1 being the value obtained in the absence of treatment.

Figure 2. The miR-23a/24-2 and miR-210 promoters are repressed by PML-RARA. A. ChIP experiments performed in NB4 cells. Chromatin was immunoprecipitated using the indicated antibodies and the enriched genomic fragments were PCR amplified using specific primers. 223-A and 223-B correspond to the two miR-223 promoter described in 20 and 17 respectively. The RARB promoter was used as a positive control. B. qPCR analyses performed on DNA immunoprecipitated in A. The negative control corresponds to a sequence
located 3.9 kb downstream the miR-23a/24-2 precursor. Fold enrichment over the negative control (no antibody) was calculated using the following formula: $2^{\Delta \Delta Ct}$ \((Ct_{input} – Ct_{IP}) – (Ct_{input} – Ct_{no\,Ab})\). C. LUC assays performed in 293T cells transfected with a firefly luciferase reporter gene driven by the miR-23a/24-2 promoter together with the control empty pcDNA3 plasmid (black histograms) or the PML-RARA expressing vector (grey histograms). A promoterless vector was used as a negative control (pGL3b). A miR-23a/24-2 promoter mutated in the predicted PML-RARA response element was also tested (mutated miR-23a/24-2 promoter). Twenty-four hours post-transfection, 293T cells were treated or not with ATRA (1μM) for 16 hours. Results are expressed as Relative Light Units (RLU), 1 representing the value obtained with the promoterless vector pGL3b in absence of PML-RARA- and ATRA-treatments.

Figure 3. miRNA modulations in ATRA-treated HL-60 and arsenic-treated NB4 cells. A. miRNAs and RARB mRNA modulations in HL-60 cells treated with ATRA 1μM for 16 hours. B. miRNAs and RARB mRNA modulations in NB4 cells treated with Arsenic Trioxide (As$_2$O$_3$) 1μM for 16 hours. RT-qPCR were performed as in Figure 1.

Figure 4. miRNA profiles of APL primary blast upon ATRA-treatment. Blast cells extracted from bone marrow of three different APL patients were cultured for 6 days with ATRA (0.1 μM). A. Viable cell counts during ATRA-treatment was determined using Trypan blue. B. Granulocytic differentiation was assessed by NBT dye reduction assay. C-G. RT-qPCR analyses specific for RARB (C), miR-23a (D), miR-210 (E), miR-223 (F), miR-181a (G). Total RNAs were extracted each day of ATRA treatment for 4 days. Results are indicated as fold change, 1 being the value obtained in the absence of ATRA treatment (0 hour post-treatment) for each patient (*, p <0.05).
**Figure 5. The miR-23a/24-2 and miR-210 promoters are regulated by RARA.** A. RT-qPCRs directed against the miR-23a, -210, and miR-223 were performed in 293T cells treated with ATRA (10 nM). Results are indicated as fold change, 1 being the value obtained in the absence of treatment. B. ChIPs performed in 293T cells using the indicated antibodies. The enriched genomic fragments were amplified using specific primers. The RARB promoter was used as a positive control. 223-A and 223-B as in Figure 2A. C. qPCR analyses performed on DNA immunoprecipitated in B. D. LUC assays performed in 293T cells transfected with promoterless pGL3b plasmid (black histograms) or the firefly luciferase reporter driven by the miR-23a/24-2 promoter (grey histograms). Cells were treated with increased doses of ATRA for 16 hours. Results are expressed as RLU, 1 representing the value obtained with the pGL3b vector in absence of treatment. E. LUC assays performed in 293T cells transfected with the firefly luciferase reporter driven by the miR-23a/24-2 promoter or the miR-23a/24-2 promoter mutated in the predicted PML-RARA response element. Cells were treated (grey histograms) or not (black histograms) with ATRA (10nM) for 16 hours. Results are expressed as RLU, 1 representing the value obtained with the pGL3b vector in absence of treatment.

**Figure 6. Functional consequences of miRNA inactivation by PML-RARA.** A. cDNA profiles obtained by microarrays (recapitulated in supplemental tables S2 and S3) were compared to the data indicated in Table 1 in order to determine the number of targeted mRNAs and the number of targeting miRNAs per mRNA. B. List of 4 down-regulated mRNAs targeted by 10 or more miRNA candidates. C. RT-qPCR directed against the uPAR mRNA performed on RNAs used in A. D. Schematic representation of the uPAR 3′UTR and the targeting miRNAs. The miRNA::mRNA hybrids are indicated.
Figure 7. miRNAs repressed by PML-RAR control crucial cancer genes. A. Luciferase assay performed in 293T cells transfected with the empty renilla luciferase reporter gene psiCHECK2 (dark histograms) or with the reporter fused to the uPAR 3’UTR (psiCHECK2-3’UTR uPAR; grey histograms). The cells were also transfected with the pcDNA3 plasmid or the PML-RARA expressing vector and treated or not with ATRA (1μM) for 16 hours as indicated. Results are expressed as RLU, 1 representing the value obtained with the empty psiCHECK2 plasmid in absence of PML-RARA and treatment. B. RT-qPCR analyses directed against the RARB mRNA and the miR-195 in 293T cells transfected and treated as in A. C. Luciferase assay performed in 293T cells transfected with the empty renilla luciferase reporter gene psiCHECK2 (dark histograms) or with the reporter fused to the EBF-3 3’UTR (psiCHECK2-3’UTR EBF-3; grey histograms). Cells were treated and results analysed as in A. D. LUC assay performed in 293T cells transfected with the psiCHECK2 or psiCHECK2-3’UTR uPAR together with specific LNA miRNA inhibitors as indicated. LNA anti-miR-32 was used as a negative control. The results are expressed as RLU, 1 being the value obtained with the empty psiCHECK2 plasmid for each LNA treatment (control).
<table>
<thead>
<tr>
<th>MicroRNA name</th>
<th>Predicted site</th>
<th>Site type</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-let-7a-2</td>
<td>TGGCCTTCTTGAAGCT</td>
<td>DR3</td>
</tr>
<tr>
<td>hsa-let-7a-3</td>
<td>TGGCCTGAGTGGACG</td>
<td>DR4</td>
</tr>
<tr>
<td>hsa-let-7c</td>
<td>TGAACCTTGTGACG</td>
<td>DR5</td>
</tr>
<tr>
<td>hsa-let-7d</td>
<td>TAACCTGTTAAATTAGGTCAC</td>
<td>ER8</td>
</tr>
<tr>
<td>hsa-mir-100</td>
<td>AGGTCAGGAGTTCAC</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-10b</td>
<td>TGTTCAAGACAGGTCAC</td>
<td>DR3</td>
</tr>
<tr>
<td>hsa-mir-130a</td>
<td>TGAACCTGTGACG</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-133b</td>
<td>AGGTCACAGAGAGGGTTCAC</td>
<td>DR3</td>
</tr>
<tr>
<td>hsa-mir-135a-1</td>
<td>AGTTCATGACG</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-146a</td>
<td>AGGTCAGGAGTTCAC</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-154</td>
<td>TAACCTCAGAACATATGGGTCAC</td>
<td>DR8</td>
</tr>
<tr>
<td>hsa-mir-183</td>
<td>mir-96</td>
<td>TGACCTTCTGGGTCAC</td>
</tr>
<tr>
<td>hsa-mir-194-2</td>
<td>mir-192</td>
<td>TAACCTCCTGAGGTCAC</td>
</tr>
<tr>
<td>hsa-mir-196a-1</td>
<td>TGACCTCGAGCACC</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-200c</td>
<td>AGGTCACAGGAGGGTTCAC</td>
<td>DR3</td>
</tr>
<tr>
<td>hsa-mir-205</td>
<td>TGACCCTCGACTGACG</td>
<td>DR4</td>
</tr>
<tr>
<td>hsa-mir-210</td>
<td>TGACCCCTGGGACG</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-217</td>
<td>TGACCTCGAGCACC</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-22</td>
<td>TGACCTCGAGCACC</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-233a</td>
<td>mir-27a</td>
<td>mir-24-2</td>
</tr>
<tr>
<td>hsa-mir-29b-2</td>
<td>TGACCCCATGACG</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-323</td>
<td>mir-758</td>
<td>TGACCTCGACTGACG</td>
</tr>
<tr>
<td>hsa-mir-331</td>
<td>TGACCTCGGGGTCAC</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-345</td>
<td>TACCCCTGGGACG</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-34a</td>
<td>AGGCCAGAGAGGGTTCAC</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-365-2</td>
<td>AGGTCAGAGAGGGTTCAC</td>
<td>DR5</td>
</tr>
<tr>
<td>hsa-mir-377</td>
<td>TGACCTCCTGACG</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-379</td>
<td>GGGGCATGACG</td>
<td>IR0</td>
</tr>
<tr>
<td>hsa-mir-380</td>
<td>AGGTCAGATGAGAGGGCA</td>
<td>DR6</td>
</tr>
<tr>
<td>hsa-mir-383</td>
<td>TGACCTCGAGCACC</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-422a</td>
<td>TGACCTGTTGACG</td>
<td>DR3</td>
</tr>
<tr>
<td>hsa-mir-455</td>
<td>GGGTCACCCAGGGCC</td>
<td>DR4</td>
</tr>
<tr>
<td>hsa-mir-497</td>
<td>mir-195</td>
<td>GGGCCAGAGGGTTCAC</td>
</tr>
<tr>
<td>hsa-mir-500</td>
<td>AGGTCACAAGAGGGTTCAC</td>
<td>DR4</td>
</tr>
<tr>
<td>hsa-mir-507</td>
<td>mir-506</td>
<td>TGACCTCCTGACG</td>
</tr>
<tr>
<td>hsa-mir-513-2</td>
<td>AGGCCAGGTGGACTGGGTTCAC</td>
<td>DR8</td>
</tr>
<tr>
<td>hsa-mir-516-1</td>
<td>AGGTCAGAGGTTCA</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-516-3</td>
<td>TGACCTCGAGCACC</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-517a</td>
<td>TGACCTGCTGACTGACG</td>
<td>DR5</td>
</tr>
<tr>
<td>hsa-mir-518a-2</td>
<td>GGGTCACCTGGGACG</td>
<td>DR4</td>
</tr>
<tr>
<td>hsa-mir-518d</td>
<td>TGACCTCGAGCACC</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-519e</td>
<td>AGGTCAGAGGTTCA</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-520b</td>
<td>AGTTCAGAGGTTCA</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-520d</td>
<td>TGACCTCGAGCACC</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-520e</td>
<td>AGGTCAGAGGTTCA</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-520f</td>
<td>AGGTCAGAGGTTCA</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-521-2</td>
<td>TGACCTCGAGCACC</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-524</td>
<td>AGTTCAGAGGTTCA</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-527</td>
<td>TGACCTCAGGCTGAGGGTTCAC</td>
<td>ER7</td>
</tr>
<tr>
<td>hsa-mir-539</td>
<td>TGCACCAAGGTGGCACC</td>
<td>DR5</td>
</tr>
<tr>
<td>hsa-mir-548a-2</td>
<td>TGACCTCCTGGCCT</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-551b</td>
<td>TGACCTTCATTCCACTT</td>
<td>DR6</td>
</tr>
<tr>
<td>hsa-mir-563</td>
<td>TGACCTTTCCCTGCACCT</td>
<td>DR5</td>
</tr>
<tr>
<td>hsa-mir-573</td>
<td>AGGTTAGGAGTTCA</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-583</td>
<td>TGGCCATGACCTTTCCCTGCACCT</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-607</td>
<td>TGAACTCCTGACCTTTCCCTGCACCT</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-612</td>
<td>TGAACTCCTGACCTTTCCCTGCACCT</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-613</td>
<td>AGGCCAGGAGTTCA</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-622</td>
<td>AGGCCAGCGGTCA</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-626</td>
<td>AGGCCAGGAGTTCA</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-645</td>
<td>TGAACCTTTCTGTGAACT</td>
<td>DR6</td>
</tr>
<tr>
<td>hsa-mir-801</td>
<td>AGGCATGACCTTTCTGTGAACT</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-9-2</td>
<td>TGAACCTTTCTGTGAACT</td>
<td>DR3</td>
</tr>
<tr>
<td>hsa-mir-92b</td>
<td>TGAACCTTTCTGTGAACT</td>
<td>DR2</td>
</tr>
<tr>
<td>hsa-mir-9-3</td>
<td>AGGCCAGGACCGGTTCACCT</td>
<td>DR5</td>
</tr>
</tbody>
</table>
Saumet et al., Figure 1
**A**

<table>
<thead>
<tr>
<th>NB4</th>
<th>Promoter</th>
<th>miR-210</th>
<th>miR-223</th>
<th>miR-23a/24-2</th>
<th>RARB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2% input</td>
<td>anti-PML</td>
<td>anti-RARA</td>
<td>anti-RXRA</td>
<td>no Ab</td>
</tr>
</tbody>
</table>

**B**

For NB4, the fold enrichment over no Ab control is shown for different promoters:
- miR-223-A
- miR-223-B
- miR-23a/24-2
- miR-210
- RARB
- negative control

**C**

For 293T, the relative light units (RLU) are shown for different conditions:
- pcDNA3
- PML-RARA

**Saumet et al., Figure 2**

32
Saumet et al., Figure 3
A

APL#1 (BCR1)

APL#2 (BCR3)

APL#3 (BCR1)

Cell viability (x100,000 cells/ml)

day 0  day 3  day 6

B

% NBT positive cells

day 0  day 3  day 6

C

RARβ

Fold change

0  24  48  72  96

hours post-treatment

D

miR-23a

Fold change

0  24  48  72  96

E

miR-210

Fold change

0  24  48  72  96

F

miR-223

Fold change

0  24  48  72  96

G

miR-181a

Fold change

0  24  48  72  96

Saumet et al., Figure 4
A

B

C

D

Saumet et al., Figure 6
Saumet et al., Figure 7
Transcriptional repression of microRNA genes by PML-RARA increases expression of key cancer proteins in acute promyelocytic leukemia

Anne Saumet, Guillaume Vetter, Manuella Bouttier, Elodie Portales-Casamar, Wyeth W. Wasserman, Thomas Maurin, Bernard Mari, Pascal Barby, Laurent Vallar, Evelyne Friederich, Khalil Arar, Bruno Cassinat, Christine Chomienne and Charles-Henri Lecellier