



## **Characterization of microRNA genes repressed by PML-RARA in Acute Promyelocytic Leukemia**



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## Summary


Micro(mi)RNAs are small non-coding RNAs that orchestrate many fundamental aspects of cell physiology and, consequently, their deregulation is often linked to cancer. Here, we studied the roles of miRNAs in Acute Promyelocytic Leukemia (APL). This malignancy is intimately associated with the generation of the oncogenic protein PML-RARA. Combining bioinformatics and transcriptomics, we identified several miRNAs targeted by PML-RARA in APL cell line and in primary blasts. Some of these miRNAs were also found physiologically regulated by retinoic acid in non-APL cells. Using comparative mRNA profiling, we identified potential targets of miRNAs repressed by PML-RARA, among those the urokinase receptor (uPAR). Together, our results reveal that PML-RARA represses miRNA genes that regulate the expression of key cancer genes.

## Significance

Our results identified several miRNA genes repressed by a major oncogene implicated in APL. These data reinforce the idea that miRNA repression can be instrumental in the initiating events of leukemia. Besides, some miRNAs were also found regulated by retinoic acid (RA) in non APL cells. These observations might be relevant in processes implicating RA, not only in normal cell physiology but also in the broad context of cancer research. In fact, one of the miRNA targets that we identified using an original approach, uPAR, is central in tumor growth and metastasis. Because RA is able to limit cancer cell invasion, our results might help develop novel therapeutic strategies aimed at limiting cancer cell evasion through pharmacological modulations of miRNAs.

## Introduction

The micro(mi)RNAs are ~22nt-long RNAs that orchestrate the translation of messenger RNAs involved in various aspects of cell biology such as cell proliferation, apoptosis and differentiation (reviewed in [1, 2, 3, 4]). The human genome encodes more than 500 miRNAs located in introns, exons or intergenic regions. Most miRNA genes are transcribed in a manner similar to coding genes but differ in their mode of post-transcriptional processing [1, 5]. It is now admitted that each cell type produces a specific miRNA repertoire regulating different cellular pathways [6]. For instance, the miR-223 has been implicated in granulopoiesis [7, 8] and negatively controls the number of granulocyte progenitors by regulating the Mef2c protein, a transcription factor promoting myeloid progenitor proliferation [8]. As a consequence of their fundamental functions, numerous studies have now directly implicated miRNAs in human pathologies, in particular cancers (reviewed in [9, 10]). The miRNAs are now considered as oncogenes or tumor suppressor genes and represent promising diagnostic as well as prognostic markers [9]. In addition, miRNAs are envisaged as novel targets of therapeutic strategies [11, 12]. The identification of miRNAs implicated in cancer might therefore help, not only get a better understanding of their implication in human diseases, but also characterize new targets of alternative chemotherapies.

Chromosomal translocations that lead to the expression of aberrant fusion proteins are frequently found in acute leukemias and many of those fusions exert dominant and negative effect on DNA-binding transcription factors or regulatory components of the transcriptional complexes [13]. For instance, the reciprocal translocation t(15;17) that fuses the retinoic acid receptor  $\alpha$  (RARA) and the promyelocytic leukemia protein (PML) is one of the most frequent translocations associated with the Acute Promyelocytic Leukemia (APL) [14]. This subtype of Acute Myeloid Leukemia (AML) is identified as AML-M3 by the French-American-British (FAB) classification and is characterized by a differentiation arrest of granulopoiesis at the promyelocytic stage (reviewed in [15]). The PML-RARA fusion protein is a transcriptional repressor that impedes the expression of several genes, among those the RA-regulated genes, through an aberrant recruitment of transcriptional repressors [16]. Consequently, these genes become insensitive to physiological doses of RA. 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 [15, 17]  The restorative effects of RA can be reproduced *in vitro*

in the NB4 cell line, which was derived from the bone marrow of an APL patient [18]. These NB4 cells provide an excellent model to study the transcriptional deregulation that arises in APL and the molecular effects of the anti-cancerous RA-based treatment [18]. Characterizing the genes whose expressions are blocked by PML-RARA is indeed crucial to understand the initiating events leading to APL and to know the molecular consequences of the anti-APL treatment.

Here, using bioinformatic and transcriptomic approaches followed by experimental validations, we identified several miRNAs targeted by PML-RARA. We further show that the repression exerted by PML-RARA could be released by ATRA even in primary blast cells. Some of the identified miRNAs were also shown to be physiologically regulated by RARA in non-APL cells. Finally, transcriptomic analyses of mRNAs modulated by ATRA in distinct APL cell lines revealed potential targets of miRNAs repressed by PML-RARA, among those the Urokinase-type Plasminogen Activator Receptor (uPAR).

## 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 [19]. 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 (SELEX) experiment [20]. 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 microRNA promoter dataset provided by Mahony *et al.* [21]). 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.

### Transcriptomic 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, a well characterised target of the RARA and PML-RARA proteins [22, 23, 24]. We then treated NB4 cells with ATRA 1 $\mu$ M 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 [25]. 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 analysed using miRNA microarrays (Fig. 1C). These chips correspond to 1067 mature human miRNAs found in the miRNA registry (<http://microrna.sanger.ac.uk/sequences/>). The oligonucleotide sequences are available on <http://www.microarray.fr> (microRNA chip). 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 [26], the miR-15b, the miR-223 and the miR-

342, 3 miRNAs previously found up-regulated in similar settings [7, 26] (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 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 conducted focusing 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 [27] and because clustered miRNAs exhibit similar expression pattern [28], 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 [20, 24]. The RARB promoter was used as a positive control [22]. We found that, while none of the two

miR-223 promoters [7, 29] were bound to PML, RARA nor 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), suggesting that these miRNAs are repressed by PML-RARA/RXRA heterodimers in APL cells, similarly to the RARB promoter. The sequence located directly upstream the miRNA precursor does not necessarily defined the miRNA promoter [7, 29] 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.* [30] 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 [31]. We observed that PML-RARA readily reduced the transcriptional activity of the miR-23a/24-2 promoters even in non APL cells (Fig. 2B). This reduction was limited by the addition of 1 $\mu$ M ATRA (Fig. 2B). In accordance with our bioinformatic 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. 2B). These results indicated that the miR-23a/24-2 polycistron is directly regulated by the oncogenic PML-RARA fusion protein through the response element identified *in silico*.

### **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 have a biological relevance and tested miRNA expression levels in primary APL blast cells extracted from the bone marrow of 3 different patients (designated patients #1, #2 and #3). RT-PCR analyses performed during diagnosis revealed that patient #2 expressed the short BCR3 isoform of PML-RARA while patients #1 and #3 expressed the long BCR1 isoform. 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. The maturation of APL cells was biochemically controlled by NitroBlueTetrazolium (NBT)-dye reduction assays at day 3 and 6 of ATRA treatment: 45% of patient #1 blast cells were NBT positive at day 6 of treatment, 58% for patient #2 and 80% for patient #3. We also controlled the induction of the prototypic RARB by ATRA (Fig. 3A). Overall, though PML-RARA isoforms were not identical, no obvious difference could distinguish the 3 patients regarding the ATRA-induced differentiation or RARB induction. RT-PCRs specific for the miR-23a, miR-210, miR-223 and miR-181a were then performed (Fig. 3B, C, D and E, respectively). The miR-181a was chosen because the expression of this miRNA was recently shown to correlate with morphological sub-class of AML [32]. We observed that the expression of the miR-23a, miR-210 and miR-223 increased

throughout the ATRA treatment although the fold inductions were not exactly comparable, the miR-223 being the less induced miRNA (Fig. 3B, C and D). The expression of the miR-181a was generally diminished by ATRA though this diminution was less pronounced at 96 hours post-treatment for patient #1, at 48 hours post-treatment for patient #2 and at 24 hours post-treatment for patient #3 (Fig. 3E). 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**

PML-RARA is thought to interfere with the functions of both parental proteins in a dominant negative manner. However, various observations identify a major gain of function of this oncogene [20, 33]. Thus, although PML-RARA binds to canonical RARA binding sites, it also recognizes wider range of DNA-target sequences that are not regulated by RARA [20]. Therefore, we were prompted to test whether the miR-210 and the miR-23a/24-2 could also be regulated by RARA in non APL cells. Classically, RAR binds DNA as heterodimer with RXR proteins, through DRs of a (A/G)G(G/T)TCA consensus with spacings of 2 or 5 [34]. In the absence of RA ligand, RAR/RXR binds transcriptional corepressors and recruits histone deacetylases leading to gene silencing. In the presence of RA, corepressors are replaced by coactivators, inducing transcriptional activation. Hence, RAR/RXR heterodimers are always bound to target sequences independently of the presence of the ligand. The promoters of the miR-210 and miR-23a/24-2 exhibited DR2 response elements, compatible with the binding of RAR/RXR (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 4A, 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. To confirm this proposal, we performed ChIP experiments, in 293T cells, to detect RA-integrating complexes physically bound to the miR-210 and the miR-23a/24-2 promoters. Using antibodies directed against both RARA and RXRA, we revealed that RARA/RXRA heterodimers could bind the miR-210 as well as the miR-23a/24-2 promoters but not the miR-223 promoter (Fig. 4B). 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 - 1 $\mu$ M) for 16 hours (Fig. 4C). We



observed that 10 nM ATRA was sufficient to induce a significant increase in the luciferase activity (Fig. 4C) 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 2B and observed that this mutant was not responsive to ATRA 10 nM (Fig. 4D). 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 suggested 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 evaluation of the PML-RARA-mediated miRNA repression**

Next, we evaluated the functional consequences of PML-RARA-mediated miRNA repression in NB4 cells and used transcriptomic approaches to identify mRNAs potentially regulated by PML-RARA-repressed miRNAs. In fact, simultaneous profiling of miRNA and mRNA expression is an appropriate strategy to identify functional miRNA targets [35]. 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 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 data 4) [31, 36]. The NB4-LR1 cells do transcriptionally respond to ATRA but do not mature [36]. In contrast, the NB4-LR2 cells show a clear defect in RA signalling [31]. 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 data 4). 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 [37]. Genes that were found up- or down-regulated in NB4 and NB4-LR1 cells but unchanged in NB4-LR2 were recapitulated in supplemental data 2 and 3 respectively. We noticed that global changes of the transcriptome were less pronounced in NB4-LR1 than in NB4 cells, again likely reflecting the activation other cellular pathways by ATRA in NB4 cells [37]. 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 Version 5

(<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 miRNAs predicted to be repressed by PML-RARA (Fig. 5A). Hence, no particular enrichment in the down-regulated gene population could be observed contrasting with our initial idea. However, translational repression orchestrated by miRNAs generally requires several targets of the same miRNA and/or distinct targeting miRNAs [3, 4, 6]. 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 data 2 and 3 (Fig. 5A). 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. 5B). 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 [38, 39], NB4 and ATRA response [40, 41].

### **Regulation of the 3'UTR of uPAR by miRNAs**

The uPAR protein play essential roles in a variety of cell functions that exploit extracellular proteolysis, adhesion and chemotaxis (reviewed in [42, 43]). Moreover, uPAR levels have been strongly correlated with the process of metastasis and over-expression of these molecules is strongly correlated with poor prognosis in a variety of malignant tumors [42]. Several studies have concluded that patients suffering with AML have high expression of uPAR that can indicate high relapse risk after therapy [38, 39]. In addition, the uPAR mRNA was specifically shown to be down-regulated by ATRA in NB4 [40] and in another leukaemic myeloid cell line, HL-60 [41]. 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. 6A). The miRBase Targets Version 5 predicted that the 3'UTR of uPAR harbored 13 sequences that might be targeted by several miRNAs predicted to be repressed by PML-RARA (Fig. 6B). 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 has slight effect on the expression of the parental *renilla* (Fig. 7A). Moreover, treatment of the transfected cells with 1 $\mu$ M ATRA abolished the effect of PML-RARA on psiCHECK2-uPAR 3'UTR (Fig. 7A). In parallel, the capacity of PML-RARA to repress the transcription of the RARB gene was evaluated by RT-qPCR (Fig. 7B). We observed that PML-RARA repressed the expression of the RARB mRNA whereas the addition of ATRA 1  $\mu$ M stopped this repression (Fig. 7B). A similar pattern was obtained for the miR-195, one of the 11 miRNAs potentially targeting uPAR mRNA and suspected to be repressed by PML-RARA (Fig. 6D and Fig. 1D). We may note that, in both cases, the addition of ATRA 1 $\mu$ M 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 modulations exerted by the uPAR 3'UTR are inversely correlated to the capacity of PML-RARA to repress transcription. Next, we verified that the post-transcriptional regulation targeting the 3'UTR of uPAR was indeed orchestrated by miRNAs. For that purpose, we transfected 293T cells with the psiCHECK2-uPAR 3'UTR vector and specific LNA miRNA inhibitors [44, 45, 46] (Fig. 7C). A functional LNA anti-miR-32 was used as a negative control [44]. 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 harbouring 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. 7C). Mixing all LNAs completely abolished the effect of the uPAR 3'UTR on *Renilla* expression (Fig. 7C), suggesting that these miRNAs acted in a coordinated manner. These results showed that the 3'UTR of uPAR is genuinely regulated by several miRNAs (i.e. the miR-194, miR-195, miR-331-5p, miR-331-3p, miR-377 and miR-622) in 293T cells. Interestingly, some of these miRNAs were suspected to be repressed by PML-RARA in APL cells (miR-194, miR-195, miR-377 and miR-622) while others could not be detected (miR-331-5p) in NB4 cells. This might indicate that distinct miRNAs could regulate uPAR expression depending on the cell type, a proposal consistent with the idea that each cell type harbours a particular miRNA repertoire [6]. Besides, our observations highlighted the importance of counting the number of targeted sequences per 3'UTR when performing simultaneous profiling of miRNAs and mRNAs to identify functional miRNA targets.

## Discussion

The role of PML-RARA in promyelocytic transformation and terminal differentiation has been extensively studied and PML-RARA appears as one of the most important oncogenes that must be inactivated in order to eradicate APL cells [14, 16, 17]. A better understanding of the biological pathways that are impaired by PML-RARA is clearly required to better understand the molecular basis of APL and to improve differentiating therapies. In that way, using bioinformatic and transcriptomic approaches, we revealed that PML-RARA is able to transcriptionally repress several miRNA genes and we formally characterized the miR-23a/24-2 and the miR-210 as authentic PML-RARA targets. 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 mediated by RARA, RA, through miRNAs, can indirectly affect post-transcriptional processes such as translation.

Overall, our microarray analyses of miRNA profiles in NB4 cells treated with ATRA and those performed by Garzon *et al.* [26] 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. In our case, the diminution of the miR-181a expression induced by ATRA was confirmed in primary APL blasts (Fig. 3E). The expression of this miRNA was recently correlated with particular subclass of AML as the miR-181a is highly expressed in AML-M1 or AML-M2 compared to AML-M4 or AML-M5 [32]. No AML-M3 sample was tested in this study [32] and we did not examine miRNA profiles in other AML subtypes. As the miR-181a is down-regulated by ATRA, the basis of the anti-APL treatment (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.* [7]. These authors functionally implicated this particular miRNA in NB4 cell maturation and concluded that the miR-223 is a positive regulator of granulopoiesis [7]. However, Johnnidis *et al.* recently demonstrated, using miR-223-deficient mice, that this miRNA is rather a negative regulator of granulopoiesis [8]. This discrepancy might merely be explained by the fact that NB4 cells are leukaemic promyelocytes with severe genetic alterations [18] that do not exactly mimic naive promyelocytes [8]. Nonetheless, 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 [47]. Interestingly, the let-7a, let-7c and let-7d miRNAs were found up-regulated by ATRA in APL cells [26] and we

predicted those miRNAs to be repressed by PML-RARA. 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-targeted mRNAs [26, 48, 49], it may be interesting to further test whether the let-7a, let-7c and let-7d are repressed by PML-RARA. Likewise, two other miRNAs, the miR-196a and the miR-10b, which could be repressed by PML-RARA (Table 1 and Fig. 1D), might be of interest. Both miRNAs are embedded in HOX clusters and regulate their expression [50, 51]. RA is known to induce a temporal program of HOX gene expression and this expression is often perturbed in leukemias [52] [53]. For instance, the deregulation of HOXB8 is associated with AML [54] and enforced expression of this gene prevents the differentiation of myeloid progenitors [55, 56, 57, 58]. The *hoxb8* mRNA is endonucleolytically cleaved by the miR-196a [50] and we found that ATRA up-regulated the expression of the miR-196a (Fig. 1). HOXB8 expression was not detected by microarrays analyses (supplemental data 2 and 3) but, 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 data 5). This expression pattern is expected for mRNAs targeted by miRNAs repressed by PML-RARA (see uPAR in Fig. 6A). The up-regulation of HOX genes in AML due to the down-regulation of miRNAs has already been suspected, though the potential link with RA-mediated transcriptional regulation has not been discussed [32, 59, 60, 61].

The miR-210 and miR-23a/24-2 loci, found here as novel PML-RARA targets, are located in intergenic regions, providing evidences that PML-RARA can also affect unsuspected chromatin regions. 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 [62]. It might be interesting to characterize the chromatin modifications associated with the PML-RARA repression of miRNA promoters and to verify whether these modifications are similar to those observed on the promoters of coding genes [62] or whether specific co-repressors might be involved. This feature may add a novel gain-of-function for PML-RARA on non-coding genes [33]. However, we showed that, similarly to coding genes, PML-RARA heterodimerizes, at least on the miR-210 and miR-23/24-2 promoters, with RXRA [20, 24]. As RXRA was recently shown to be an essential factor in APL pathogenesis [24], these findings might draw attention to the importance of the transcriptional repression of miRNAs in the development of APL. In that sense, it has been proposed that the uPA system, which appears here as one of the targets of the miRNAs potentially repressed by PML-RARA,

contribute to AML blast cell dissemination and disease invasion [38]. Likewise, high expression of uPAR was reported in AML samples and patients with higher proportions of uPAR-positive cells had a significant lower remission rate after chemotherapy and a higher risk for relapse [39]. The authors even proposed that uPAR could be a relevant prognostic marker of AML independent of the karyotype [39]. Malignant APL promyelocytes were also suspected to be the source of high uPA activity which may be responsible for the bleeding disorder observed in APL patients [63]. Curiously, Mustjoki *et al.* have shown that 13-*trans* retinoic acid increased uPAR mRNA and protein levels in NB4 cells [64, 65]. However, the authors showed that the global effect of RA was a decrease in proteolytic activity due to, at least, the activation of plasminogen activator inhibitors (PAI) [64, 65]. Although we and others have found that ATRA rather decreases uPAR mRNA in NB4 (Fig. 6A and [40]) even in HL-60 cells [41], our results are consistent with the observation that the overall outcome of ATRA is a decrease in uPA activity [65]. But, in addition to PAI [65], we revealed that the miRNA pathway could also be implicated in this process. uPAR plays in fact important roles in tumor growth and metastasis and overexpression of this molecule is strongly correlated with poor prognosis in a variety of malignant tumors [66, 67, 68]. This protein is therefore envisaged as an attractive target of novel anti-cancerous therapies [42, 43, 69, 70, 71, 72]. In this context, we may note that RA treatment is able to limit the invasion of various cancer cells [73, 74, 75] and we found here a link between miRNAs that are potentially regulated by RA and uPAR expression. Hence, it would be interesting to further examine the potential modulations of these miRNAs during tumor cell invasion and to check whether they are implicated in uPAR regulation in that context. Pharmacological modulations of uPAR by RA might indeed be beneficial for the therapeutic limitation of cancer cell evasion.

## Experimental Procedures

**Cell lines, primary blast cells and ATRA-treatment.** The human APL NB4 and ATRA-resistant NB4-LR1 and NB4-LR2 cell lines were cultured as previously described [76] and treated with 1  $\mu$ M ATRA (Sigma) for 16 hours. 293T cells were maintained in DMEM (Gibco-BRL) supplemented with 2mM L-glutamine, 100  $\mu$ g/ml penicillin, 50  $\mu$ g/ml streptomycin, 240 mM HEPES and 5% heat-inactivated foetal 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 (AML3) 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.

### Microarrays miRNAs

(Thomas MAURIN)

### Microarrays cDNA

(Guillaume VETTER)

**DNA constructs and Luciferase Assays.** The sequence corresponding to the miR-23a/24-2 promoter (2046 bp upstream miR-23a precursor) was cloned into the *Bgl*III/*Hind*III restriction sites of pGL3b vector (Promega). All sets of primers are indicated in Supplemental data 1. The predicted PML-RARA response element of the miR-23a/24-2 promoter was mutagenized using the QuickChange Site-Directed Mutagenesis procedure (Stratagene). The 3'UTR of uPAR was fused to the *renilla* gene using the *Xho*I/*Not*I restrictions sites of the psiCHECK2 vector (Promega). The pcDNA3 vector encoding PML-RARA is described in [31]. Transfections of 293T cells were performed using Lipofectamine 2000 (Invitrogen). When indicated, overnight treatment (1  $\mu$ M ATRA, 16 hours) was realized 24 hours after transfection. Luciferase assays were performed, in all cases, 48 hours post-transfection 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.

LNA/DNA mixed oligonucleotides used as miRNA inhibitors were provided by Sigma-Prologo and sequences are indicated in supplemental data 1. Indicated results are means of at least three independent experiments.

**Quantitative RT-PCR.** Total RNAs were extracted using Trizol (Invitrogen) for NB4 and 293T cell lines or using RNAlplus (QBiogen) for primary blast cells. RTs were realized using the SuperScript II RT (Invitrogen) and oligodT(N) for RARB, uPAR, HOXB8 and GAPDH or specific stem-loop oligonucleotides indicated in supplemental data 1 for miRNAs. PCRs were realized using SYBR Green PCR Master Mix (Roche) and the sets of primers are indicated in supplemental data 1. The expression of GAPDH was used to normalize the expressions of RARB and HOXB8. The miRNA-specific RT-qPCR protocol was adapted from [77] to SYBR Green technology. Indicated results are means of at least three independent experiments.

**Chromatin Immunoprecipitation.** ChIP experiments were performed using a standard protocol as described in [78]. Briefly, the cross-linked chromatin was sonicated and immunoprecipitated overnight at 4°C by using antibodies against PML, RARA or RXRA (10µg) (Santa-Cruz). Immunoprecipitated DNA was used as template for PCR using sets of primers indicated in supplemental data 1.



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## 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 (1  $\mu$ M) 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 or not with 1  $\mu$ M ATRA for 16 hours. **B.** RT-qPCR analysis of RAR $\beta$  mRNA expression in NB4 cells upon ATRA treatment. Results are indicated as fold induction, 1 being the cycle threshold (Ct) obtained in the absence of treatment. **C.** Table of miRNAs modulated by ATRA. See the Materials and Methods section for details. **D.** miRNA-specific RT-qPCRs directed against the miR-10b, 133b, -146a, -181a, -181b, -194, -195, -196a, -210, -223, -23a, -331, -377, and miR-622 performed in untreated (black histograms) and ATRA-treated NB4 cells (grey histograms). The asterisk (\*) indicates an absence of amplification. Results are indicated as fold induction, 1 being the Ct obtained in the absence of treatment.

**Figure 2. The miR-23a/24-2 and miR-210 promoters are repressed by PML-RARA.** **A.** Chromatin immunoprecipitation (ChIP) experiments performed in NB4 cells. Chromatin was immunoprecipitated using anti-PML, anti-RARA and anti-RXRA antibodies, and the enriched genomic fragment was amplified using specific primers for each miRNA promoter (see supplemental data 1 for positions and sequences of each primers used). 223-A and 223-B correspond to the two miR-223 promoter described in [29] and [7] respectively. The RAR $\beta$ 2 promoter was used as a positive control. **B.** Luciferase 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 ( $\Delta$ PML-RARARE) was also tested. Twenty-four hours post-transfection, 293T cells were

treated or not with ATRA (1  $\mu$ M) 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 profiles of APL primary blast upon ATRA-treatment.** Blast cells extracted from bone marrow of three different APL patients (designated here APL#1, #2, and #3) were cultured for 4 days with ATRA (100  $\mu$ M). Total RNA were extracted each day of treatment and analysed by RT-qPCR. **A.** RT-qPCR analysis of the RARB mRNA during ATRA-treatment of APL blast cells. **B, C, D, and E.** miRNA-specific RT-qPCR directed against miR-23a, miR-210, miR-223 and miR-181a, respectively. Results are indicated as fold induction, 1 being the Ct obtained in the absence of treatment.

**Figure 4. 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 a physiological dose of ATRA (10 nM). Results are indicated as fold induction, 1 being the Ct obtained in the absence of treatment. **B.** Chromatin immunoprecipitation experiments performed in 293T cells using anti-RARA and anti-RXRA antibodies. The enriched genomic fragment was amplified using specific primers for each miRNA promoter (see supplemental data 1 for positions and sequences). The RAR $\beta$ 2 promoter was used as a positive control. 223-A and 223-B as in Figure 2A. **C.** Luciferase 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 (10, 100 or 1000 nM) for 16 hours. Results are expressed as RLU, 1 representing the value obtained with the pGL3b vector in absence of treatment. **D.** Luciferase 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 ( $\Delta$ PML-RARARE). 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 5. Comparison of miRNAs versus mRNA profiles in APL cell lines upon ATRA-treatment.** **A.** cDNA profiles obtained by microarrays (recapitulated in supplemental data 2 and 3) 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.

**Figure 6. uPAR is modulated by ATRA in NB4 cells.** **A.** RT-qPCR directed against the uPAR mRNA performed on RNAs used in Figure 5A. **B.** Schematic representation of the uPAR 3'UTR and the targeting miRNAs. The miRNA::mRNA hybrids are indicated.

**Figure 7. uPAR is regulated by PML-RARA and the miRNA pathway.** **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 (1  $\mu$ M) 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 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 (referred in the figure to as control).

Saumet et al., Table 1

MicroRNA	name	Predicted site	Site type
hsa-let-7a-2		TGGCCTTCTTGAAC	DR3
hsa-let-7a-3		TGGCCTGAGCTGACCG	DR4
hsa-let-7c		TGAACTATTGTTGAACG	DR5
hsa-let-7d		TAACTGTAAATTAGGTCA	ER8
hsa-mir-100		AGGTCAGGAGTTCA	DR2
hsa-mir-10b		TGTTACAGACAGGTCA	DR3
hsa-mir-130a		TGACCCAGTGAAC	DR2
hsa-mir-133b		AGGCCAAGAAGTTCA	DR3
hsa-mir-135a-1		AGTTCATGACCT	IR0
hsa-mir-146a		AGGTCAGGAGTTCA	DR2
hsa-mir-154		TGACCTCAGAATCATGGCCT	DR8
hsa-mir-183	Imir-96	TGACCTTCTGGCCT	DR2
hsa-mir-194-2	Imir-192	TAACTCTCTGAACC	DR3
hsa-mir-196a-1		TGAACTCCTGACCT	DR2
hsa-mir-200c		AGGTCAGCGAGGGGTCA	DR5
hsa-mir-205		TGACCTGCTCTGGACT	DR4
hsa-mir-210		TGACCCCTTGACCC	DR2
hsa-mir-217		TGAACTCCTGACCT	DR2
hsa-mir-22		TGAACTGGCCCTGACCC	DR5
hsa-mir-23a	Imir-27	AGGCCAGGAGTTCA	DR2
hsa-mir-29b-2		TGACCCCATGAACC	DR2
hsa-mir-323	Imir-758	TGACCTGCACTGCACC	DR4
hsa-mir-331		TGAACTCCTGGCCT	DR2
hsa-mir-345		TACCCTGGTGACCC	DR2
hsa-mir-34a		AGGCCAGGAGTTCA	DR2
hsa-mir-365-2		AGGGCAGTAGAGGTCA	DR5
hsa-mir-377		TGAACCATGTGACCG	DR3
hsa-mir-379		GGGGCATGAAC	IR0
hsa-mir-380		AGGTCAGTGAAGAGGCCA	DR6
hsa-mir-383		TGAACTCCTGACCT	DR2
hsa-mir-422a		TGGCCTTGTGACCT	DR3
hsa-mir-455		GGGTCACCCAGGGCCA	DR4
hsa-mir-497	Imir-195	GGGCCAGGAGGTCA	DR2
hsa-mir-500		AGGTCAACAAAGTTCA	DR4
hsa-mir-507	Imir-506	TGAACTCCTGGCCT	DR2
hsa-mir-513-2		AGGCCAGGTGCAGTGGGTCA	DR8
hsa-mir-516-1		AGGTCAGGAGTTCA	DR2
hsa-mir-516-3		TGAACTCCTGACCT	DR2
hsa-mir-517a		TGACCTGGTCATGCACC	DR5
hsa-mir-518a-2		GGGTCACCTGAGGTCA	DR4
hsa-mir-518d		TGAACTCCTGACCT	DR2
hsa-mir-519e		AGGTCAGGAGTTCA	DR2
hsa-mir-520b		AGTTCAGGAGTTCA	DR2
hsa-mir-520d		TGAACTCCTGACCT	DR2
hsa-mir-520e		AGGTCAGGAGTTCA	DR2
hsa-mir-520f		AGGTCAGGAGTTCA	DR2
hsa-mir-521-2		TGAACTCCTGACCT	DR2
hsa-mir-524		AGTTCAGGAGTTCA	DR2
hsa-mir-527		TGCCCTCCAGCCTGGGTCA	ER7
hsa-mir-539		TGCACCAAGTTTGACCT	DR5
hsa-mir-548a-2		TGACCTCCTGGCCT	DR2
hsa-mir-551b		TGACCTTCATTTTAACCT	DR6
hsa-mir-563		TGACCTTTCCCTGCACT	DR5
hsa-mir-573		AGGTTAGGAGTTCA	DR2
hsa-mir-583		TGGCCCATGACCT	DR2
hsa-mir-607		TGAACTCCTGACCT	DR2
hsa-mir-612		TGAACTCCTGACCT	DR2
hsa-mir-613		AGGCCAGGAGTTCA	DR2
hsa-mir-622		AGGCCAGGCGGTCA	DR2
hsa-mir-626		AGGCCAGGAGTTCA	DR2
hsa-mir-645		TGAACCCTATTGTGAACT	DR6
hsa-mir-801		AGGTCACAAGGTCA	DR2
hsa-mir-9-2		TGAACCTTATGAACT	DR3
hsa-mir-9-3		AGGCCAGCCACGGTTCA	DR5
hsa-mir-92b		TGAACCCCTGACCT	DR2

## **Supplemental data**

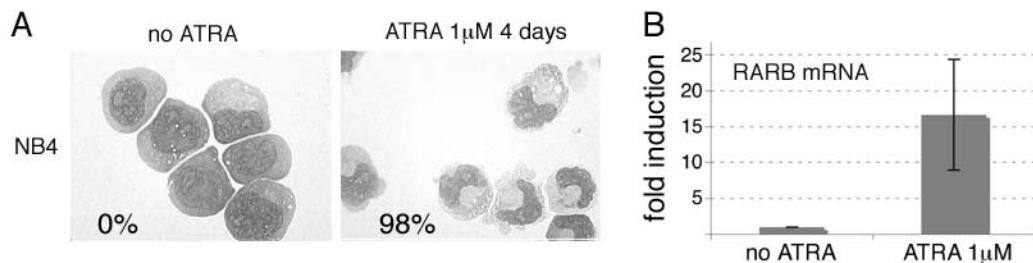
**Supplemental data 1.** Set of primers and LNAs used in this study.

**Supplemental data 2.** cDNA microarray results : up-regulated mRNAs upon ATRA in APL cell lines.

**Supplemental data 3.** cDNA microarray results : down-regulated mRNAs upon ATRA in APL cell lines.

**Supplemental data 4. A.** Morphology of MGG-stained of NB4-LR1 and NB4-LR2 cells treated or not with ATRA (1  $\mu$ M) for 4 days. Cell maturation was monitored by NBT-dye reduction assay and the percentage of NBT positive cells at 4 days of treatment is indicated (bottom left). **B.** NB4-LR1 and NB4-LR2 cells were treated with ATRA (1  $\mu$ M) for 16 hours. RNAs used in Figure 5A were also analyzed by RT-qPCR directed against RARB mRNA. Results are indicated as fold induction, 1 being the Ct obtained in absence of treatment.

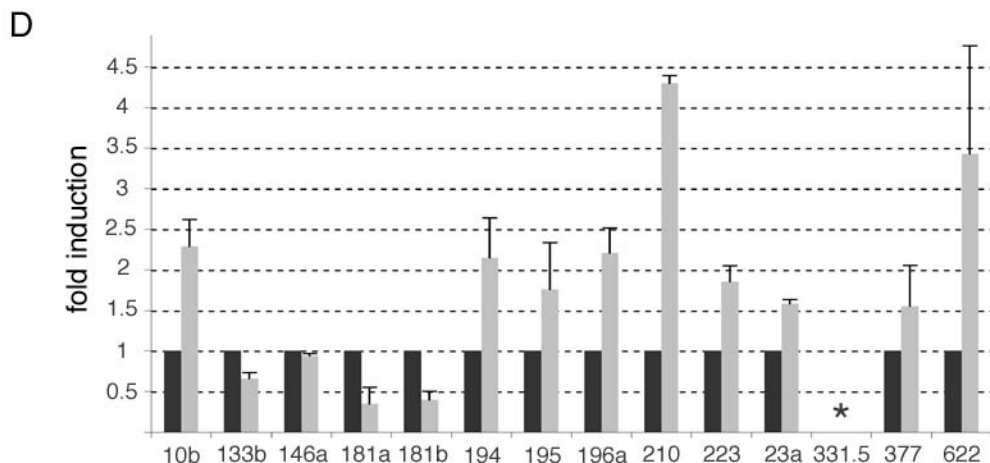
**Supplemental data 5.** RT-qPCR directed against the HOXB8 mRNA performed on RNAs extracted from NB4, NB4-LR1 and NB4-LR2 treated or not with 1 $\mu$ M ATRA for 16 hours.



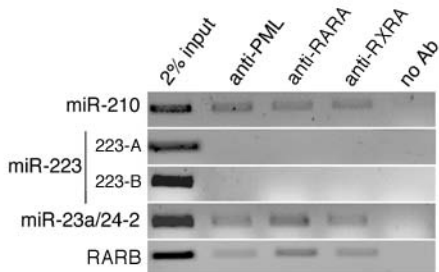
**C**

up-regulated miRNAs	log ratio
hsa-miR-143	0.64
hsa-miR-15b	0.41
hsa-miR-210	0.67
hsa-miR-223	1.05
hsa-miR-30c-1	0.48
hsa-miR-339	0.23
hsa-miR-342	0.53
hsa-miR-373	0.43

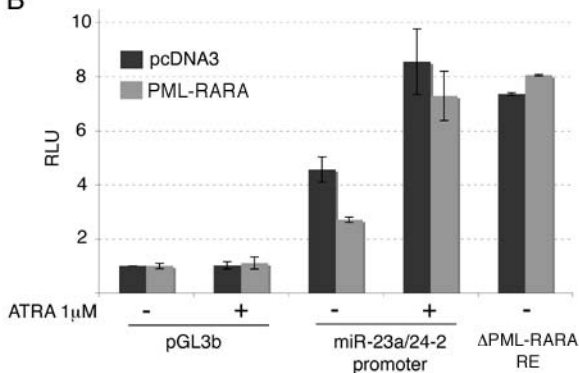
down-regulated miRNAs	log ratio
hsa-miR-106a	-1.04
hsa-miR-129-1	-0.46
hsa-miR-181a-2	-0.28
hsa-miR-181b-1	-0.40
hsa-miR-181c	-0.21
hsa-miR-181d	-0.40
hsa-miR-20b	-0.49
hsa-miR-513-2	-0.32



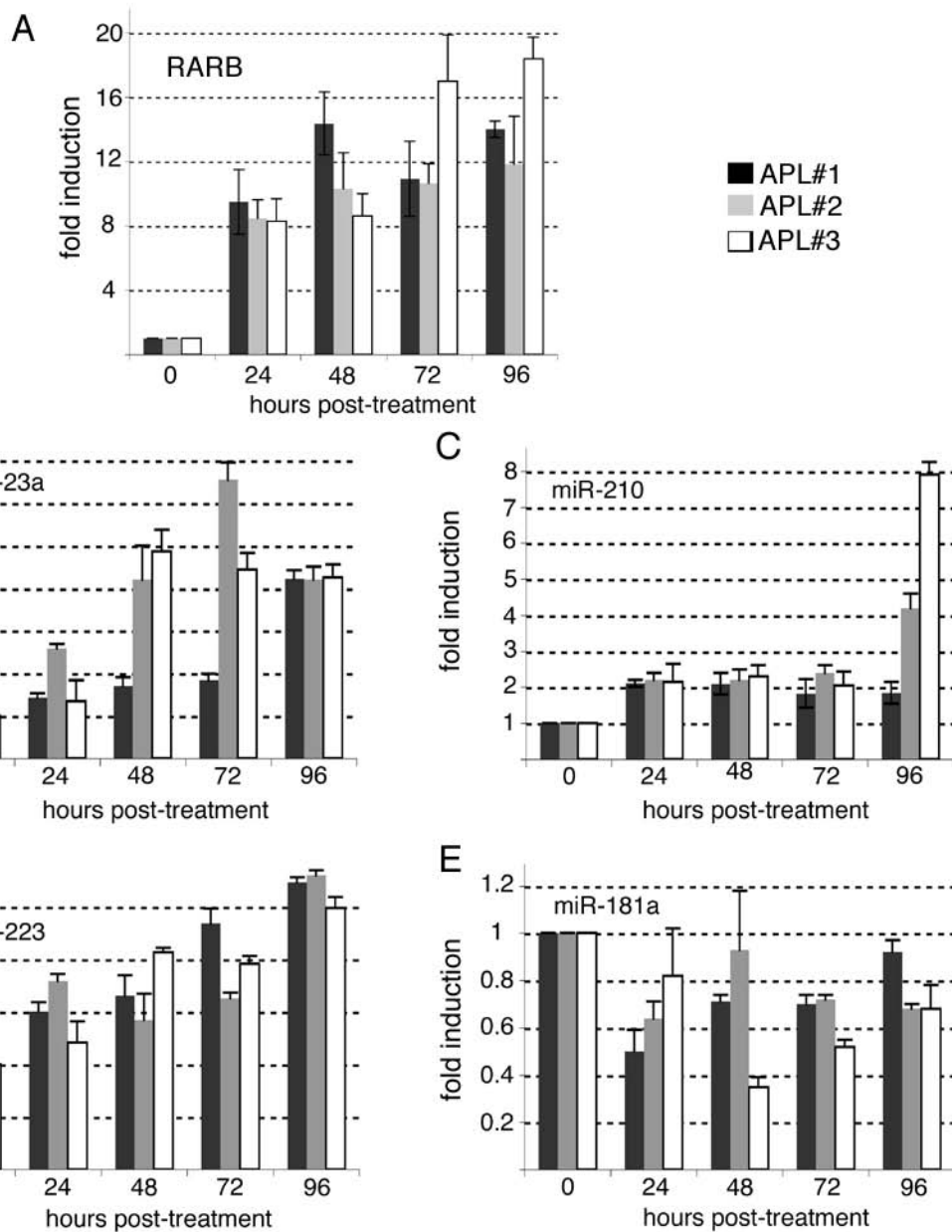
A



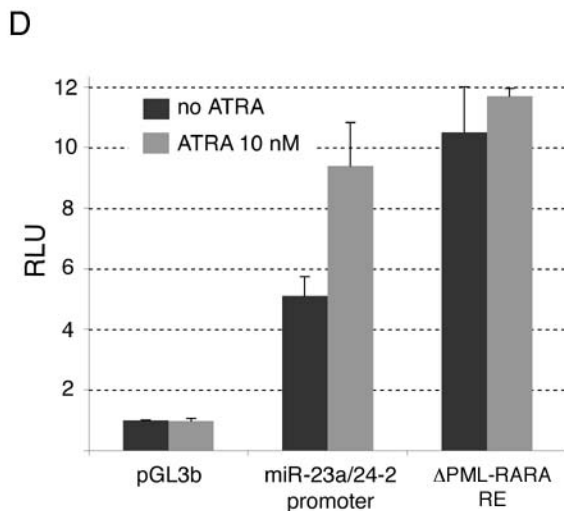
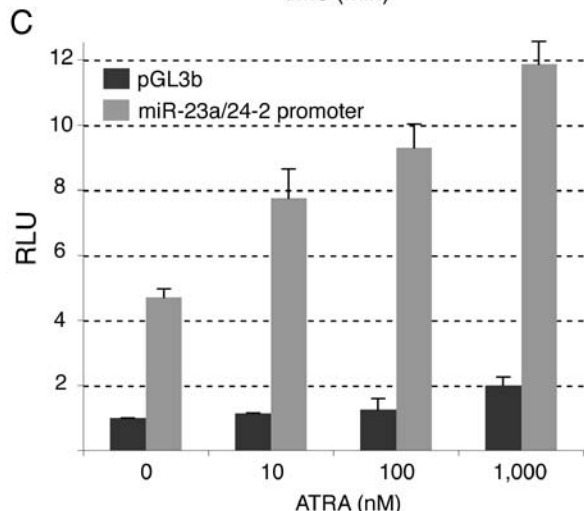
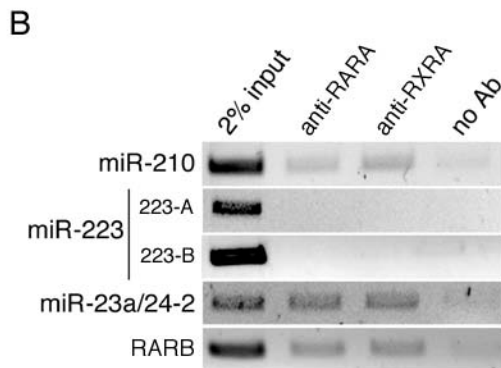
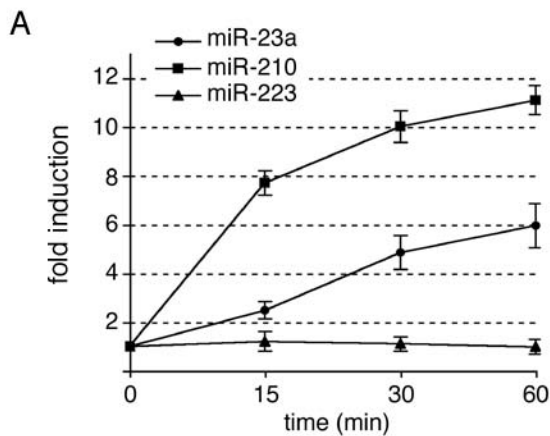
B



Saumet et al., Figure 2

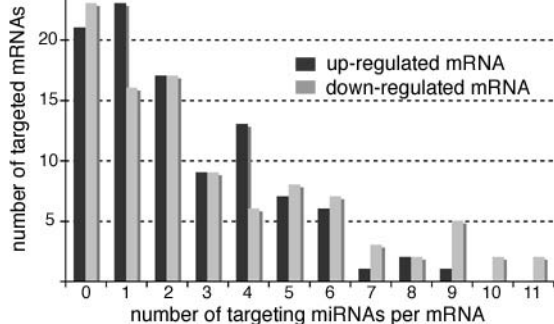


Saumet et al., Figure 3



Saumet et al., Figure 4

A

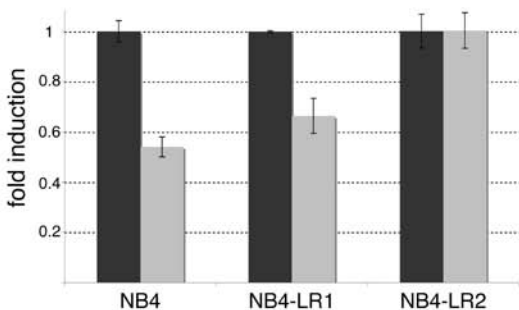


B

number of targeting miRNAs	ensembl reference	gene symbol
10	ENST00000272521	
10	ENSG00000133460	SLC2A11
11	ENST00000221264	PLAUR, CD87, uPAR
11	ENST00000264874	RIOK1



**A**       no ATRA       ATRA 1 $\mu$ M - 16 hours



**B**

hsa-miR-331-3p  
hsa-miR-133b  
hsa-miR-622  
hsa-miR-377  
hsa-miR-146a  
hsa-let-7a, d, c  
hsa-miR-377  
hsa-miR-133b  
hsa-miR-194  
hsa-miR-195  
hsa-miR-331-5p

**3'UTR UPAR**

**hsa-miR-331-3p:**

aagauCCUAUCCGGGUCCCCg  
|| :| :||:|  
gccctGGCTGGATCCGGGGGa

**hsa-miR-146a:**

uuGGGUACCUUAAGUCAAGAGu  
:| :||: ||||:|  
ccTCTCTGGGCCTCAGTTTTCc

**hsa-miR-133b:**

aUCGACCAACUUC-----CCUGGUUu  
||||| ||||| |:|  
aAGCTGGAGGAAGGCCGTGGGCCAAt

**hsa-let-7a:**

UUGAUUAUGUUGGAUGAUGGAGU  
|:|:|: || | :|:|:|  
AGCTATGAAAACAGCTATCTCA

AUCGACCAACUUC-----CCUGGUUu

|:|:|:| | :|:|:|  
TGGCTGGATCCGGGGGACCcct

**hsa-let-7d:**

UUGAUACGUUGGAUGAUGGAGa  
|:|:|:| || | :|:|:|  
AGCTATGAAAACAGCTATCTCa

**hsa-miR-622:**

CGAGGUUGGAGUCGUCUGAca  
||||| | | | |||||  
GCTCCAGCCCTACAGACTtg

**hsa-let-7c:**

UUGGUAUGUUGGAUGAUGGAGU  
|:|:|:| || | :|:|:|  
AGCTATGAAAACAGCTATCTCA

**hsa-miR-377:**

uguUUUCAACGGAAACACACUa  
|:| |||| | |||||  
tacAGACTTGC---TGTGTGAc

**hsa-miR-194:**

aGGUG-UACCUCAACGACAAUGu  
||:| :| | ||||:|:|:|  
gCCGCTGTTGTGTTGTTGTTATt

uguuuucacggaAACACACUa

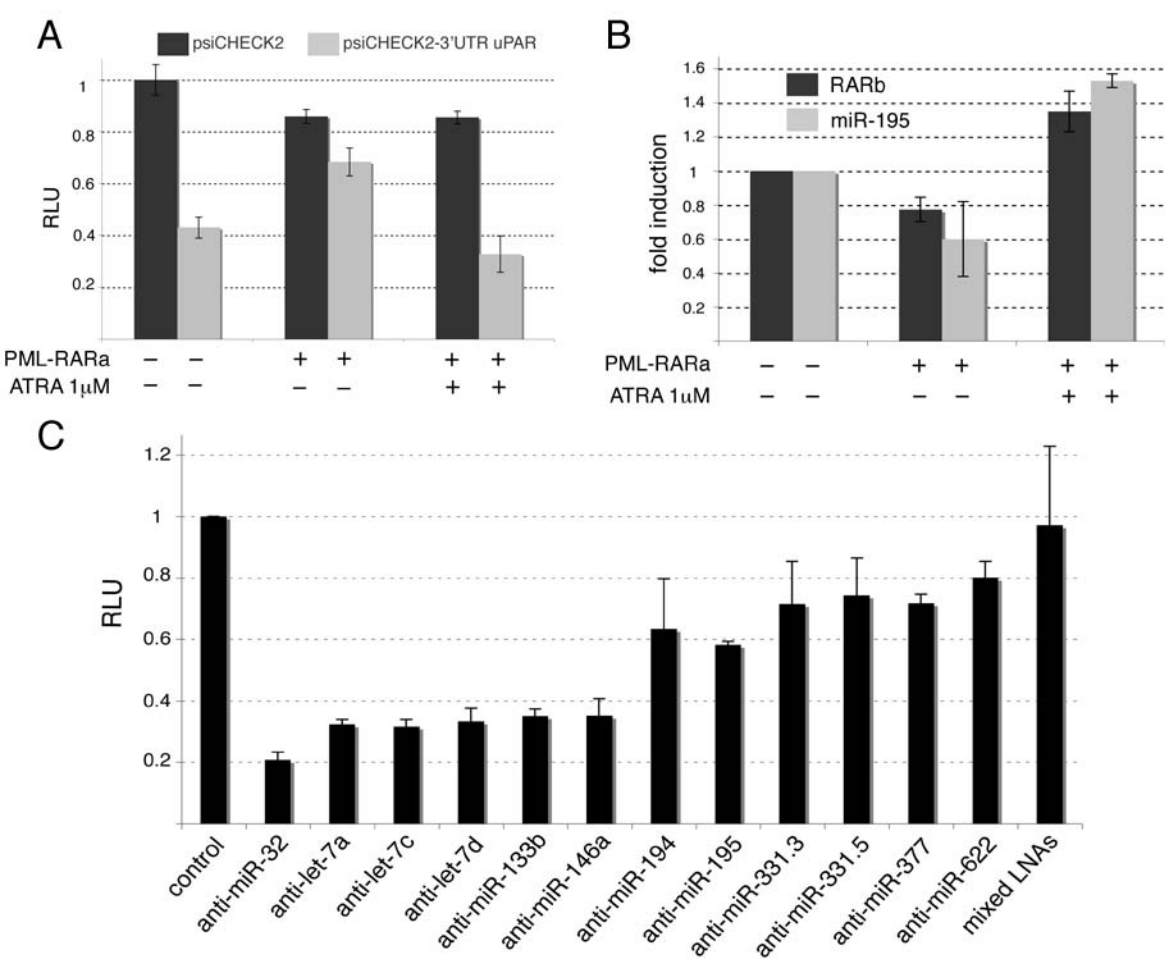
|||||||  
ctatctcacaagTTGTGTGAa

**hsa-miR-195:**

CGGUUAUAAAGACACGACGAU  
|||: |:|:| :|:|:|  
GCCGCTGTTGTGTTGTTGTTA

**hsa-miR-331-5p:**

ccUAGGGACCCUGGAUGGAUc  
||: |:| :||:|:|  
atATTATTTATTTTATACTTAc



Saumet et al., Figure 7

## Saumet et al., supplemental data 1

### Cloning miR-23a/24-2 promoter in pGL3b

sense	GGAAGATCTAGAAGCTCCACTCACTAAAGACTTAGGGC
antisense	CccaagcttCCCCAGGAACCCCAGCCGGCCGTG

### Cloning 3' UTR uPAR in psiCHECK2

sense	ccgCTCGAGACCTGAAATCCCCCTCTCTG
antisense	atagtttaGCGGCCGCCACTGGTACAAAATCTTTATGTAAG

### $\Delta$ PML-RARA RE in miR-23a/24-2 promoter

sense	GTATTGCGCAGGCTGGTCTCTCGAGCCTGGCCTCAGGTGACTCT
antisense	GAGAGTCACCTGAGGCCAGGCTCGAGAGACCAGCCTGCGCAATA

### Chromatin IP

#### RARB promoter

sense	GGAAAGAAAACGCCGGCTTG
antisense	GCCCAGACAAACCCTGCTCG

#### miR-23a/24-2 promoter

sense	GGAAGATCTGGAGCAAGGATTTGAACCTTGGG
antisense	cccaagcttCCCCAGGAACCCCAGCCGGCCGTG

#### miR-223 promoter (223-A)

sense	AAAGATCTCTGTCAGTGGAGTGGTGCC
antisense	AAGCTAGCAAGGTCAGCTGGGAGTTGG

#### miR-223 promoter (223-B)

sense	CTCCTGTCATTCTCACAATAAC
antisense	CCAAGAGCTTCTGTGGGGAAG

#### miR-210 promoter

sense	agggcggtgagtttaggg
antisense	GCGCTGAAAACGTGTAAATC

### RT-qPCR

#### RARB

sense	ttgtgttcacctttgccaac
antisense	cggttcctcaaggtcctg

#### uPAR

sense	acaccaccaaatagcaacga
antisense	ccccttgacgtgtaacac

#### HOXB8

sense	CTTTGTAATGACCAAGGTACCG
antisense	GAGAGAGAGAGAAGGGAGACAGA

#### GAPDH

sense	GAAGGTGAAGGTCGGAGTC
-------	---------------------

antisense            GAAGATGGTGATGGGATTTTC

miR-23a

RT GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACggaat

PCR sense            GCGatcacattgccagg

The same antisense primer was used for all PCR : GTGCAGGGTCCGAGGT

miR-10b

RT GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACCACAAAT

PCR sense TCGGGTACCCTGTAGAACCG

miR-133b

RT GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACTagctg

PCR sense            CGGtttggtccccttcaac

miR-146a

RT GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACaacc

PCR sense            GGCGGtgagaactgaattcca

mir-181a

RT GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACactcac

PCR sense            CGGaacattcaacgctgtcg

miR-181b

RT GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACcccacc

PCR sense            GGCGGaacattcattgctgtc

miR-194

RT GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACTccaca

PCR sense            GCGGtgtaacagcaactcca

miR-195

RT GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACgccaat

PCR sense            GGCGGtagcagcacagaaat

miR-196a

RT GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACcccaac

PCR sense            CGGCGGtaggtagtttcatgtt

miR-210

RT GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACTcagcc

PCR sense            GGctgtgctgtgtgacagc

miR-223

RT GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACggggta

PCR sense            GCGGgtgcagtttgtcaaa

miR-331-5p

RT GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACgggatcc

PCR sense            GCGGctaggtatggtccag

miR-377

RT GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACacaaaa

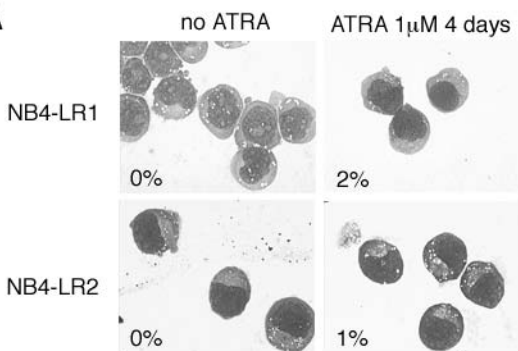
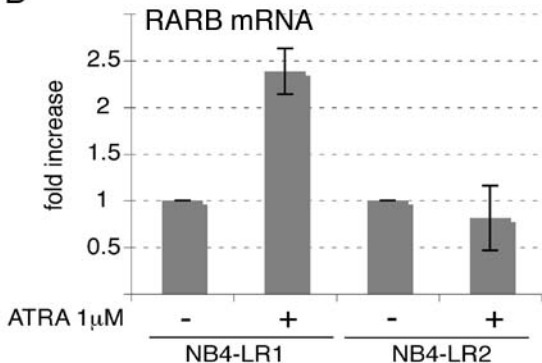
PCR sense            GCGGatcacacaaaggcaac

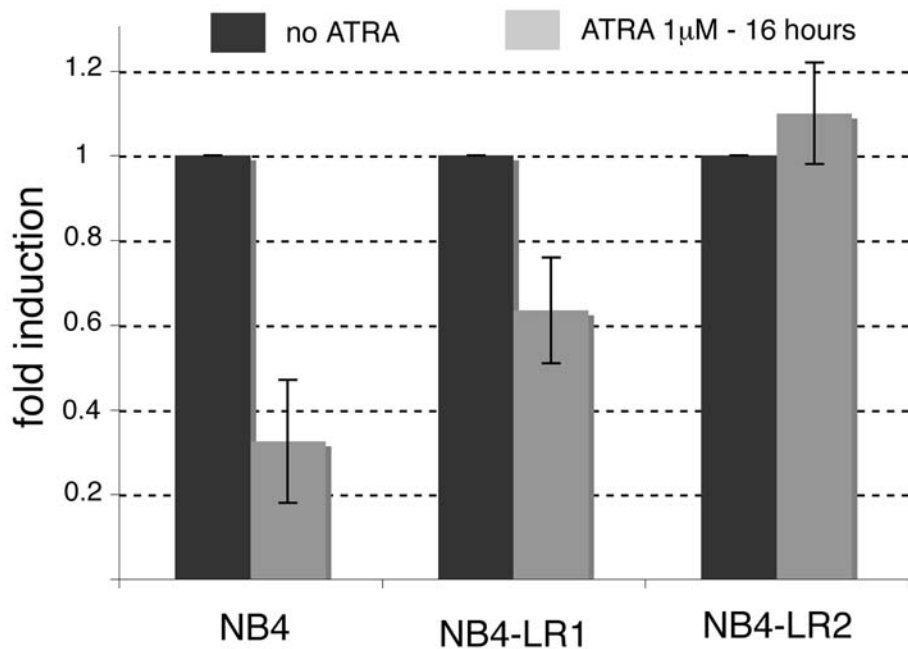
miR-622  
RT GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACgctcca  
PCR sense GCGGacagtctgctgaggt

### **mixed LNA/DNA oligonucleotides**

N+ indicates an LNA base

hsa-miR-32	
antisense	g+c+a+a+cttagtaatgtgca+a+t+a+
hsa-miR-331-3p	
antisense	t+tct+agg+ata+ggc+cca+ggg+gc
hsa-miR-331-5p	
antisense	gg+atc+cct+ggg+acc+ata+cct+ag
hsa-miR-133b	
antisense	ta+gct+ggt+tga+agg+gga+cca+aa
hsa-miR-622	
antisense	g+ctc+caa+cct+cag+cag+act+gt
hsa-miR-377	
antisense	ac+aaa+agt+tgc+ctt+tgt+gtg+at
hsa-miR-146a	
antisense	aa+ccc+atg+gaa+ttc+agt+tct+ca
hsa-let-7a	
antisense	aa+cta+tac+aac+cta+cta+cct+ca
hsa-let-7d	
antisense	aa+cta+tgc+aac+cta+cta+cct+ct
hsa-let-7c	
antisense	aa+cca+tac+aac+cta+cta+cct+ca
hsa-miR-194	
antisense	tc+cac+atg+gag+ttg+ctg+tta+ca
hsa-miR-195	
antisense	g+cca+ata+ttt+ctg+tgc+tgc+ta

**A****B**



Saumet et al., Supp data 5