Title:

A network including PU.1, Vav1 and miR-142-3p sustains ATRA-induced differentiation of acute promyelocytic leukemia cells - a short report

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Keywords:

Acute promyelocytic leukemia; ATRA; miR-142-3p; PU.1; Vav1.

Abstract

Purpose Reduced expression of miR-142-3p has been found to be associated with the development of various subtypes of myeloid leukemia, including acute promyelocytic leukemia (APL). In APL-derived cells, miR-142-3p expression can be restored by all-*trans* retinoic acid (ATRA), which induces the completion of their maturation program. Here, we aimed to assess whether PU.1, essential for ATRA-induced gene transcription, regulates the expression of miR-142-3p in APL-derived cells and, based on the established cooperation between PU.1 and Vav1 in modulating gene expression, to evaluate the role of Vav1 in restoring the expression of miR-142-3p.

Methods ATRA-induced increases in PU.1 and Vav1 expression in APL-derived NB4 cells were counteracted with specific siRNAs, and the expression of miR-142-3p was measured by quantitative real-time PCR (qRT-PCR). The recruitment of PU.1 and/or Vav1 to the regulatory region of *miR-142* was assessed by quantitative chromatin immunoprecipitation (Q-ChIP). Synthetic inhibitors or mimics for miR-142-3p were used to assess whether this miRNA plays a role in regulating the expression of PU.1 and/or Vav1.

Results We found that the expression of miR-142-3p in differentiating APL-derived NB4 cells is dependent on PU.1, and that Vav1 is essential for the recruitment of this transcription factor to its cis-binding element on the *miR-142* promoter. In addition, we found that in ATRA-treated NB4 cells miR-142-3p sustains agonist-induced increases in both PU.1 and Vav1.

Conclusions Our results suggest the existence of a Vav1/PU.1/miR-142-3p network that supports ATRA-induced differentiation in APL-derived cells. Since selective regulation of miRNAs may play a role in the future treatment of hematopoietic malignancies, our results may provide a basis for the development of new therapeutic strategies to restore the expression of miR-142-3p.

1 Introduction

In acute promyelocytic leukemia (APL), the M3 subtype of myeloid acute leukemia (AML), the t(15;17)-associated *PML-RARA* gene fusion results in a block of myeloid differentiation at the promyelocyte stage [1]. Although the treatment of APL patients includes arsenic trioxide and chemotherapy, the use of all-*trans* retinoic acid (ATRA) constitutes, at present, the only example of a successful differentiation therapy of human cancer in which tumor cells, instead of being destroyed, are induced to complete their maturation to neutrophils [2].

One of the crucial proteins in the myeloid maturation program activated by ATRA in APL-derived cells is Vav1, the sole member of the Vav family of proteins that is expressed only in haematopoietic cells, where it plays an essential role in both maturation and physiology-related functions [3, 4]. In malignant promyelocytes, Vav1 interacts with a number of signaling molecules in both cytoplasmic and nuclear compartments, where it participates in interconnected networks regulating different aspects of agonist-induced maturation along granulocytic and monocytic lineages [5]. In ATRA-treated APL-derived NB4 cells, a specific role of Vav1 in modulating gene expression has been reported, i.e., it can control the nuclear amount of proteins involved in mRNA production and stability [6]. A direct interaction of Vav1 with transcription factors that play a crucial role in the maturation of myeloid cells has also been observed, next to its participation in DNA-protein complexes [7, 8]. During ATRA-induced differentiation of NB4 cells, Vav1 interacts with PU.1 and regulates the recruitment of this transcription factor to the DNA sequence that drives the expression of the surface antigen CD11b [9].

In both normal and malignant myelopoiesis PU.1 activity is considered to be crucial, since it regulates the expression of growth factor receptors, adhesion molecules, mediators of intracellular signalling cascades and nuclear proteins, including Vav1 [10-12], and various miRNAs, some of which have been related to myeloid leukemogenesis [13-15]. Among the latter, miR-142-3p has been found to be deregulated in more than 90% of all myeloid leukemias, including APL [16], and

itôs up-regulation has been reported to be characteristic for agonist-induced monocytic and granulocytic differentiation of AML-derived cells [17], suggesting a possible role as target in the treatment of AML.

Since it has been found that in a murine model of myeloid leukemia PU.1 plays a critical role in initiating miR-142 expression [17], we aimed to assess the involvement of this transcription factor in regulating miR-142-3p expression during ATRA-induced completion of the differentiation program of APL-derived promyelocytes. Based on the established cooperation between PU.1 and Vav1 in modulating gene expression, the involvement of Vav1 in regulating miR-142-3p expression was also investigated.

2 Materials and methods

2.1 Cell culture and differentiation

All reagents were purchased from Sigma Chemicals Co. (USA) unless otherwise indicated. The NB4 cell line was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and was cultured in RPMI-1640 medium (Gibco Laboratories, USA) supplemented with 10% FBS (Gibco Laboratories, USA) at 37°C in a humidified atmosphere containing 5% CO_2 in air. The cell density was maintained between 5×10^5/ml and 1.5×10^6 /ml. The cells were monthly tested for mycoplasma and other contaminations and quarterly subjected to cell identification using single-nucleotide polymorphism (SNP) typing.

Granulocytic differentiation of NB4 cells was induced by the administration of 1 εM ATRA for 0 to 96 h. The degree of granulocytic differentiation was monitored by nuclear staining with 4Ñj 6-diamidino-2-phenylindole (DAPI) and by evaluating CD11b surface expression after direct staining with a PE-conjugated anti-CD11b antibody, as previously reported [18]. The cell

morphology was evaluated using conventional bright-field microscopic analysis of May-Grunwald-Giemsa stained cytospins using a Carl Zeiss Axiophot 100 system (Göttingen, Germany).

2.2 RNA interference assays and modulation of miR-142-3p

Exponentially growing NB4 cells were transfected with a mixture of small interfering RNAs (siRNAs, Santa Cruz Biotechnology, USA) targeting the mRNAs of PU.1 or Vav1, using a previously reported procedure [9]. As a control for transfection efficiency, which was always higher than 60%, a non-silencing fluorescein-labelled duplex RNA (Qiagen, USA) was used.

For the modulation of miR-142-3p expression, transient transfections were carried out with 30 nM synthetic inhibitors or mimics (mirVana miRNA, Life Technologies, USA), as previously reported [9]. Random sequences were transfected as negative controls. After a 5 h transfection period, cells were treated with ATRA for 72 h and, subsequently, subjected to immunochemical, quantitative real-time PCR and chromatin immunoprecipitation assays.

2.3 Immunochemical assay

Total cell lysates were separated in 7.5% polyacrylamide denaturing gels and blotted onto nitrocellulose membranes (GE Healthcare Life Science, USA). The membranes were incubated with antibodies directed against PU.1, Vav1 (Santa Cruz Biotechnology, USA) and ß-Tubulin (Sigma, USA) as previously reported [9]. Chemiluminescence-derived bands were acquired using an ImageQuantÊ LAS 4000 biomolecular imager (GE Healthcare, USA), and densitometry analyses were performed using Image Quant TL software (GE Healthcare, USA).

2.4 Quantitative real-time PCR assay

For quantitative real-time PCR (qRT-PCR) high-quality small RNAs from NB4 cells were extracted using a Mirvana miRNA Isolation Kit (Life Technologies, USA) according to the manufacturer's instructions. 10 ng RNA was subjected to single stranded cDNA synthesis using a TaqMan

MicroRNA Reverse Transcription Kit (Life Technologies, USA) according to the manufacturer's instructions. The cDNAs obtained were employed as templates for quantitative Real-Time PCR-based miR-142-3p expression measurements using TaqMan MicroRNA Assays (Life Technologies, USA). miRNA expression levels were determined using the $2^{-\Delta\Delta Ct}$ method and normalized to U6 snRNA (Life Technologies, USA). Thermal cycling and fluorescence detection were performed according to the manufacturerôs instructions, using a Bio-Rad CFX96™ sequence detection system (Bio-Rad Laboratories, USA) and the data were analysed using dedicated software (Bio-Rad Laboratories, USA).

2.5 Quantitative chromatin immunoprecipitation assay

Quantitative chromatin immunoprecipitation (Q-ChIP) experiments were performed using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY, USA) according to the manufacturer's instructions and as previously reported [19]. Briefly, $2x10^6$ untreated and treated NB4 cells were fixed with 1% formaldehyde to cross-link the DNA with proteins, lysed and sonicated to obtain DNA fragments of 200-1000 bp. After pre-clearing with salmon sperm DNA/protein-A agarose beads, the samples were subjected to immunoprecipitation using antibodies specific for PU.1, Vav1 or (negative control) IgG (Santa Cruz Biotechnology, USA) at 4 °C overnight. Next, the beads were washed, protein/DNA complexes eluted and cross-links reversed by heating at 65 °C overnight. After protein digestion, DNA was recovered using a PCR purification kit (Promega, USA) in 50 ml elution buffer.

Quantitative PCR of a 248 bp DNA fragment, encompassing the putative PU.1 binding site located at -199/-193 bp from the transcriptional start in the human *miR-142* promoter and showing 100% homology with the PU.1 consensus sequence located in the murine promoter [15], was performed in triplicate using an iTaq Universal SYBR green SuperMix on a Bio-Rad CFX96™ Real-time detection system (Bio-Rad Laboratories, USA). The primers used were: Fw: 5Nj CTAGTCTCTACCTGAGTGTCTC-3Njand Rev: 5NjCTTGTGGCTTCCTAAGATCC -3Nj Input

corresponding to 1% of the total sonicated DNA was used as a positive control.

2.6 Statistical analyses

Statistical analyses were performed using the two-tailed Student's t-test for unpaired data. *P* values < 0.05 were considered statistically significant.

3 Results and discussion

In recent years, miRNAs, short non-coding RNAs that target specific mRNAs, have been found to play crucial roles in gene expression (de)regulation during normal and abnormal (cancer) development [insert refs. Cell. Oncol. 38: 307-317, 2015; Cell. Oncol. 38: 173-181, 2015; Cell. Oncol. 37: 167-178, 2014], including leukemogenesis [20-22; insert ref. Cell. Oncol. 38: 93-109, 2015]. Among them, miR-142-3p has been found to act as a key regulator of the normal differentiation program of both lymphoid and myeloid cells [23, 24], whereas its reduced expression has been correlated with the development of the M1 to M5 subtypes of AML [16]. In AML M1, M2 and M3-derived cells, it has been found that miR-142-3p expression is restored upon agonist-induced completion of their differentiation programs along the monocytic and granulocytic lineages [17], indicating that high levels of this miRNA are necessary for regulating the differentiation of myeloid cells. In particular, it has been found that an increase in miR-142-3p expression accompanies, and its forced up-regulation promotes, granulocytic maturation induced by ATRA in APL (AML-M3)-derived NB4 cells [17], suggestive of a specific role of this miRNA in overriding the differentiation block of these malignant promyelocytes by ATRA. Even though the molecular mechanisms underlying the expression of specific miRNAs are still poorly defined, transcriptional regulation is considered to be a key event. Up-regulation of miR-142-3p has been observed to result from over-expression of miR-223 in K562 cells, and to involve C/EBPβ and

LMO2 in the miR-223-C/EBPβ-LMO2-miR-142 pathway, which plays a pivotal role in hematopoiesis [25].

For the expression up-regulation of miR-142-3p during ATRA treatment of APL-derived cells, PU.1 serves as a good candidate since it appears to be essential for ATRA-induced gene transcription [26] and to be required for initiating *miR-142* expression in murine myeloid leukemia cells [15]. In order to assess the putative role of PU.1 in determining miR-142-3p expression during ATRA treatment of human APL-derived cells, this transcription factor was down-modulated in NB4 cells exposed to ATRA for 72 h, corresponding to the peak of the miRNA level in this experimental model (Fig. 1a). By doing so, we found that, when the expression of PU.1 was silenced by siRNAs during ATRA administration (Fig. 1b), the up-regulation of miR-142-3p by the agonist (ATRA) was completely neutralized (Fig. 1c), which is indicative of a decisive role for this transcription factor in up-regulating miR-142-3p levels during granulocytic differentiation of APL-derived promyelocytes.

Cis-regulatory elements for PU.1, C/EBPβ and Runx1 have been found to be present within the *miR-142* promoter. In addition, it has been found in a murine AML model that PU.1 absence is sufficient to abrogate miR-142-3p expression, despite the presence of Runx1 and C/EBPβ, thus indicating a predominant role for PU.1 in the regulation of this miRNA [15]. Despite its synergy with other transcription factors, interaction of PU.1 with the *miR-142* promoter was found to be critical for the initiation of miR-142 expression in this model [15]. To address the question whether also in human malignant promyelocytes PU.1 is recruited to the regulatory region of *miR-142*, NB4 cells treated with ATRA (72 h) were subjected to Q-ChIP assays using primers able to amplify a region corresponding to the putative PU.1 binding site located at -193 bp within the human *miR-142* promoter (Fig 2a). By doing so, we found that the low amount of chromatin immunoprecipitated by the anti-PU.1 antibody under control conditions was substantially increased after ATRA treatment, which is indicative of a direct recruitment of PU.1 to the *miR-142* promoter during ATRA-induced differentiation of APL-derived cells (Fig. 2b).

It is well known that PU.1 acts as a transcriptional regulator in concert with a variety of other factors, including transcription factors, non-DNA binding cofactors and chromatin remodelling factors [27]. It has e.g. been reported that in ATRA treated NB4 cells a collaboration between PU.1 and IRF-9 regulates the expression of miR-342 which, in turn, is involved in the granulocytic differentiation program activated by ATRA in APL-derived cells [14]. Previously, we have shown that during the differentiation of NB4 cells PU.1 requires the cooperation of Vav1, a multifunctional signalling protein involved in the expression of proteins induced by ATRA in malignant promyelocytes [5]. Here, we show that Vav1 affects the expression of miR-142-3p, since its siRNA-mediated silencing in NB4 cells exposed to ATRA (Fig. 1b) totally counteracted the agonist-induced increase in the expression of this miRNA (Fig. 1c). Since we previously found that Vav1 interacts with PU.1 and regulates its presence in DNA/protein complexes within the PU.1 consensus region of the *CD11b* promoter in APL-derived cells [9], we set out to assess a possible relationship between these two proteins during miR-142-3p expression. Using Q-ChIP we found that, as previously reported for CD11b [9], an adequate amount of Vav1 is crucial for the recruitment of PU.1 to its consensus sequence within the *miR-142* promoter (Fig. 2b). These data underscore the wide-ranging role of Vav1 in promoting the access of PU.1 to its recognition sites within DNA, which likely serves to modulate its transcriptional activity during ATRA-induced differentiation of APL-derived promyelocytes. In order to explore the involvement of Vav1 in the transcription of *miR-142* in further detail, Q-ChIP assays were performed using an anti-Vav1 antibody. By doing so, we failed to observe any significant amounts of DNA under all experimental conditions used (data not shown), thereby excluding a direct participation of Vav1 in the DNA/protein complexes recruited by ATRA to the PU.1 recognition motif within the *miR-142* promoter.

From these data we conclude that PU.1 acts as a major transcription factor that regulates the expression of miR-142-3p during ATRA-induced granulocytic differentiation of malignant promyelocytes. The interaction of PU.1 with the *miR-142* promoter appears to be dependent on the

presence of Vav1 which, thus, is essential for the ATRA-induced up-regulation of this miRNA. Although the molecular mechanisms underlying miR-142-3p expression deregulation in other hematological malignancies remain to be identified, down-modulated levels of PU.1 and/or Vav1 may be considered as crucial events.

Increased miR-142-3p expression has been observed at different stages of granulocytic differentiation, and up-regulation of miR-142-3p has been found to characterize the granulocytic maturation of CD34+ precursor cells [28]. In both normal myeloid differentiation and AML development miR-142-3p acts, at least in part, through direct suppression of cyclin T2 (CCNT2) and the TGF-activated kinase 1/MAP3K7 binding protein 2 (TAB2) [17]. Since miR-142-3p promotes ATRA-induced granulocytic differentiation of NB4 cells [17] and both PU.1 and Vav1 are crucial for the granulocytic maturation of malignant promyelocytes [5, 26], the implications of miR-142-3p expression in ATRA-dependent modulation of these two proteins was investigated using a specific inhibitor (Fig. 3a). Through staining and cytofluorimetrical evaluation of the myeloid marker CD11b, we first confirmed that adequate miR-142-3p expression levels are required to accomplish the ATRA-induced maturation program of APL-derived cells. In NB4 cells, in which miR-142-3p was silenced during ATRA treatment, we observed a lower number of poly-lobular nuclei (Fig. 3b, 3c), a higher nuclear/cytoplasmic ratio (Fig. 3c) and a significantly lower cell surface expression of CD11b (Fig. 3d). Immunochemical analyses of lysates from NB4 cells under the same experimental conditions showed that miR-142-3p inhibition partially counteracted the ATRA-induced increases in both PU.1 and Vav1 expression (Fig. 3e, 4a). On the other hand, and confirming our previous data [9], we found that silencing of PU.1 during ATRA treatment substantially reduced the agonist-induced expression of Vav1 (Fig. 1b, 4a), suggesting that the effects of miR-142-3p on Vav1 expression may be mediated by PU.1. After exogenous over-expression of miR-142-3p in both control and ATRA-treated cells we failed to observe any significant effects on the expression levels of both PU.1 and Vav1 (data not shown). These data indicate that a sole over-expression of miR-142-3p is not sufficient to modulate the two proteins

but, instead, highlight the existence of a positive feedback-loop in NB4 cells treated with ATRA (summarized in Fig. 4b), in which PU.1 and Vav1 regulate miR-142-3p which, in turn, is essential for the agonist-induced expression of both proteins. Based on literature and target gene prediction data, neither PU.1 nor Vav1 appear to be directly related to miR-142-3p downstream targets. Validated targets of miR-142-3p in other cell models include *ADCY9* [23], *CD133* [28], *IL-6* [29] and *RAC1* [30], suggesting that the myeloid differentiation promoting effect of this miRNA may be mediated by a number of indirect targets. The positive correlation between the expression levels of PU.1, Vav1 and miR-142-3p that we observed in ATRA-treated NB4 cells suggests that this miRNA may be part of an ATRA-activated machinery responsible for the removal of suppressor protein(s) that negatively affect PU.1 activity.

We conclude that our data provide evidence for the existence of a Vav1/PU.1/miR-142-3p network that sustains ATRA-induced granulocytic differentiation of promyelocytic cells and outline the unprecedented contribution of Vav1 in regulating the expression of specific miRNAs. Since the selective regulation of miRNAs is regarded as an option for the future treatment of hematopoietic malignancies, our results may be instrumental for the design of potential strategies to combat AML via restoring the expression of miR-142-3p.

Conflict of Interest statement

The authors declare that they have no conflicts of interest.

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Figure legends

Fig. 1 (**a**) qRT-PCR analysis of miR-142-3p levels in NB4 cells grown in the presence of ATRA for the indicated times. The values are shown as fold changes relative to the untreated condition. (**b**) Western blot analysis using the indicated antibodies of NB4 cells in which PU.1 or Vav1 were down-regulated by specific siRNAs under control conditions and during 72 h ATRA treatment. Ctrl siRNAs, non-silencing scramble siRNAs; PU.1 siRNAs, siRNAs specific for PU.1; Vav1 siRNAs, siRNAs specific for Vav1. (**c**) qRT-PCR analysis of miR-142-3p levels in NB4 cells in which PU.1 or Vav1 were silenced under control conditions and during 72 h ATRA treatment. The values are shown as fold changes relative to Ctrl siRNAs, which were set at 1. All values represent the means of 3 separate experiments ± SD. The asterisks indicate statistically significant differences ($p < 0.05$).

Fig. 2 (**a**) Schematic representation of the PU.1 binding site within the human *miR-142* promoter. The arrow indicates the transcriptional start site. (**b**) Analysis of *in vivo* recruitment of PU.1 to the human *miR-142* promoter by chromatin immunoprecipitation (ChIP). Protein-DNA complexes were cross-linked *in vivo* by formaldehyde in NB4 cells in which Vav1 was down-regulated during 72 h ATRA treatment. Chromatin fragments were subjected to immunoprecipitation with an antibody directed against PU.1 or IgG (negative control). After cross-link reversal, the co-immunoprecipitated DNA was amplified by PCR using a primer pair spanning a 248 bp region encompassing the -193/-199 bp PU.1 consensus binding sequence. The data are shown as percentage of the input (genomic DNA collected before immunoprecipitation). All values represent the means of 3 separate experiments ± SD. The asterisk indicates a statistically significant difference ($p < 0.05$).

Fig. 3 (**a**) qRT-PCR analysis of miR-142-3p expression in NB4 cells transfected with a miR-142-

3p inhibitor (Anti-miR-142-3p) or with a scramble inhibitor (Ctrl anti-miR) and induced to differentiate with ATRA for 72 h. The data are presented by setting the ATRA value at 1. NB4 cells under the same experimental conditions were subjected to nuclear staining with DAPI (**b**), May-Grunwald-Giemsa staining (**c**), cytofluorimetrical analysis of CD11b expression by direct staining with a PE-conjugated anti-CD11b antibody (**d**) and immunochemical analysis with the indicated antibodies (**e**). The asterisks indicate statistically significant differences ($p < 0.05$). Bar: 20 µm.

Fig. 4 (**a**) PU.1 and Vav1 levels deduced from densitometry analyses of Western blot-derived bands from ATRA treated NB4 cells in which PU.1, Vav1 or miR-142-3p were down-regulated. The values were normalized using ϐ-Tubulin as internal control for protein loading and represent fold changes relative to untreated conditions, which were set at 1. Ctrl, non-silencing scramble sequences; PU.1 siRNAs, siRNAs specific for PU.1; Vav1 siRNAs, siRNAs specific for Vav1; Anti-miR-142-3p, inhibitor of miR-142-3p. All values represent the means of 3 separate experiments performed in triplicate ± SD. The asterisks indicate statistically significant differences ($p < 0.05$). (**b**) Schematic representation of the PU.1/Vav1/miR-142-3p network induced by ATRA in NB4 cells.

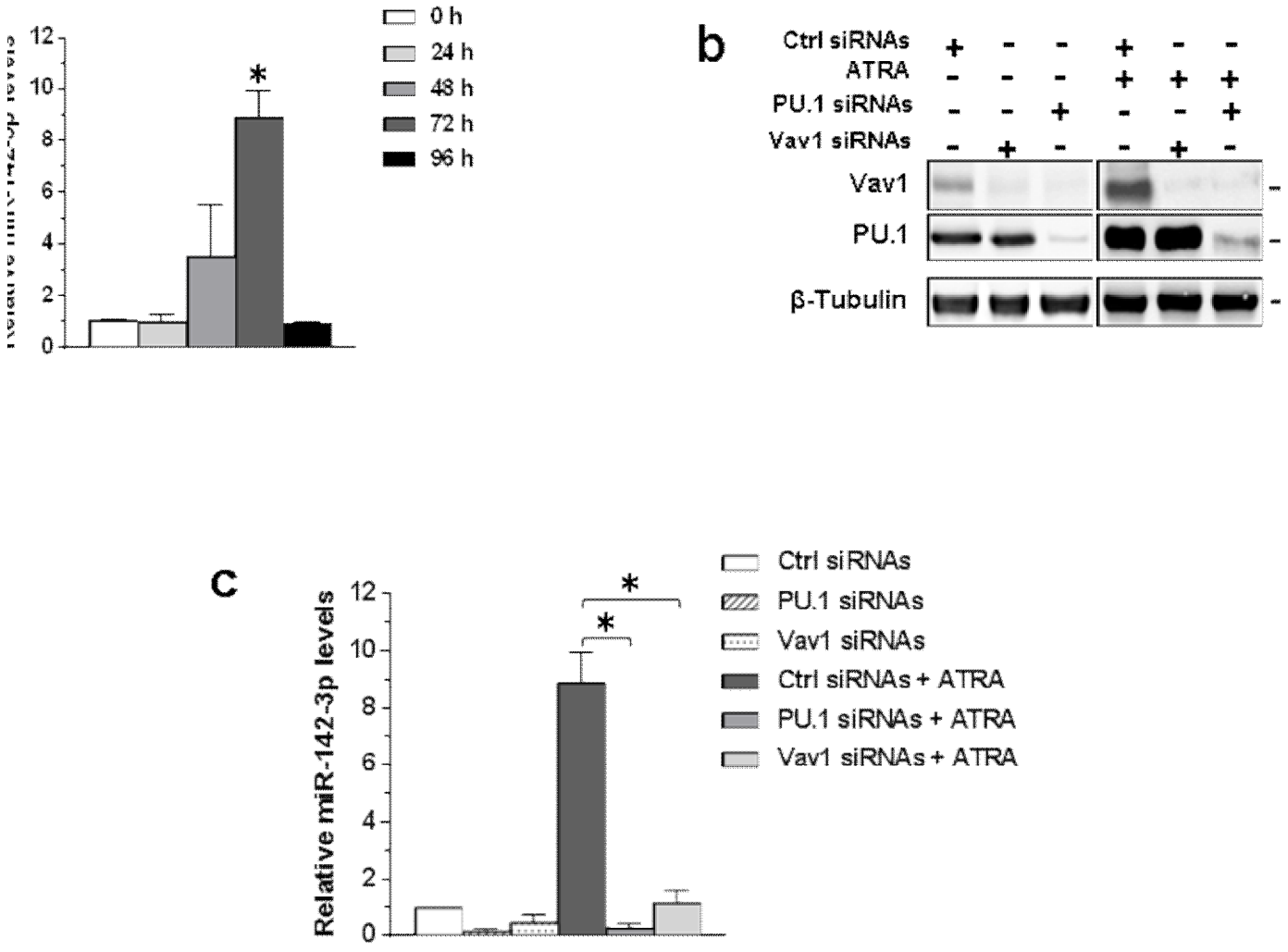

0 h
24 h
48 h
72 h
96 h
*
b
Ctrl siRNAs
ATRA
PU.1 siRNAs
Vav1 siRNAs
Vav1
PU.1
β-Tubulin
c
Relative miR-142-3p levels
Ctrl siRNAs
PU.1 siRNAs
Vav1 siRNAs
Ctrl siRNAs + ATRA
PU.1 siRNAs + ATRA
Vav1 siRNAs + ATRA

Figure 1

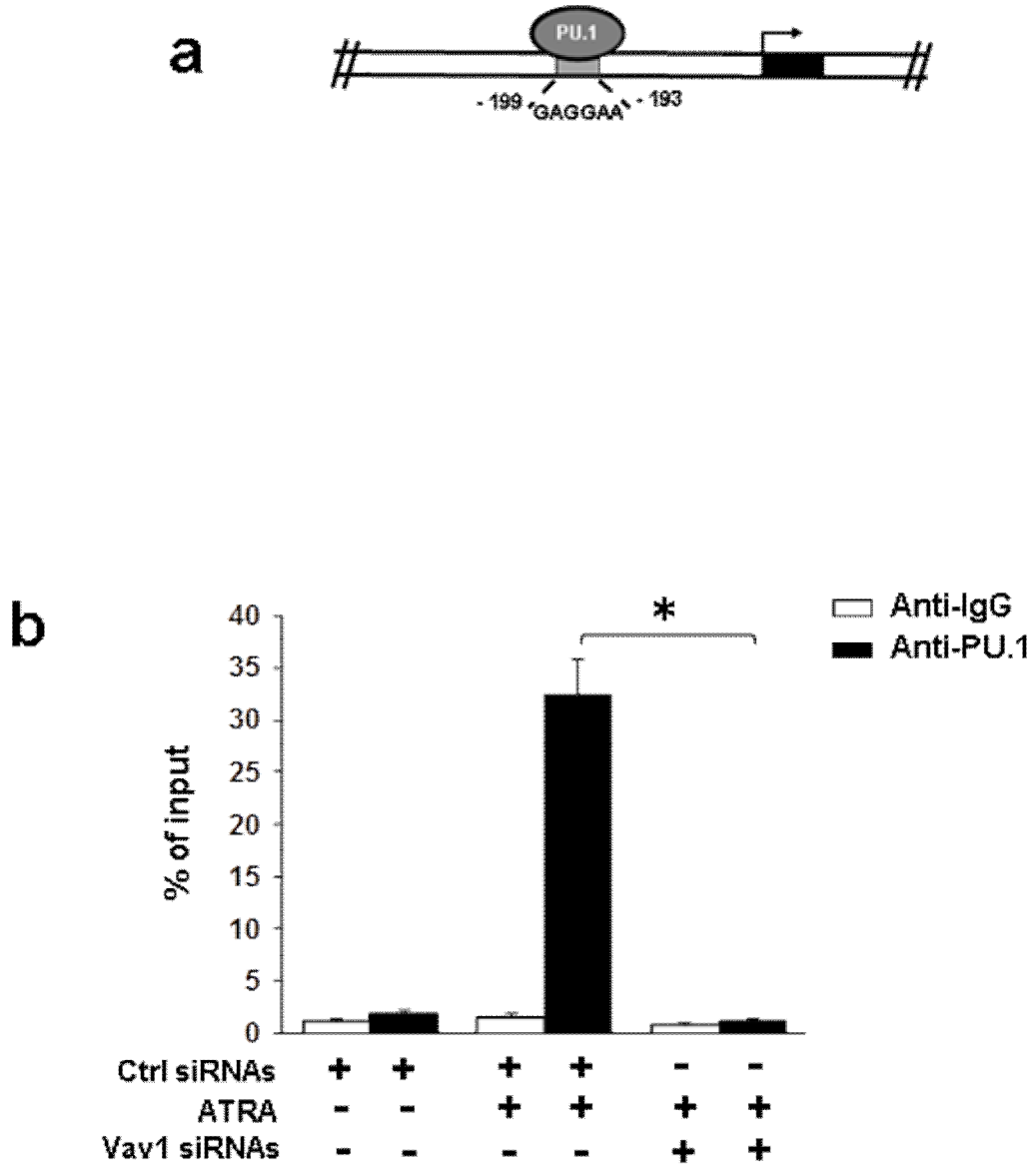

a
PU.1
- 199
GAGGAA
- 193
b
Anti-IgG
Anti-PU.1
*
% of input
40
35
30
25
20
15
10
5
0
Ctrl siRNAs + + + + - -
ATRA - - + + + +
Vav1 siRNAs - - - - + +

Figure 2

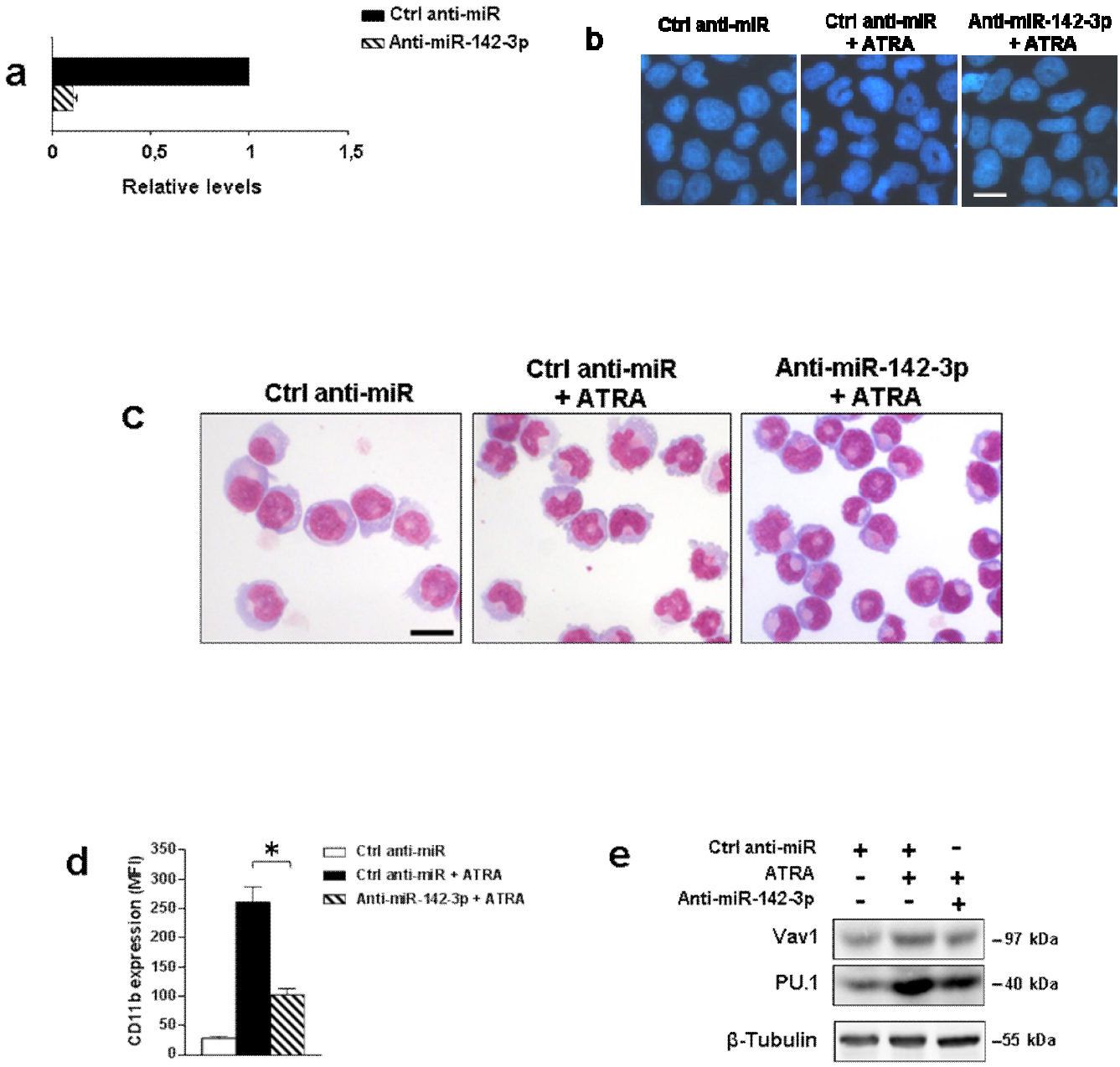
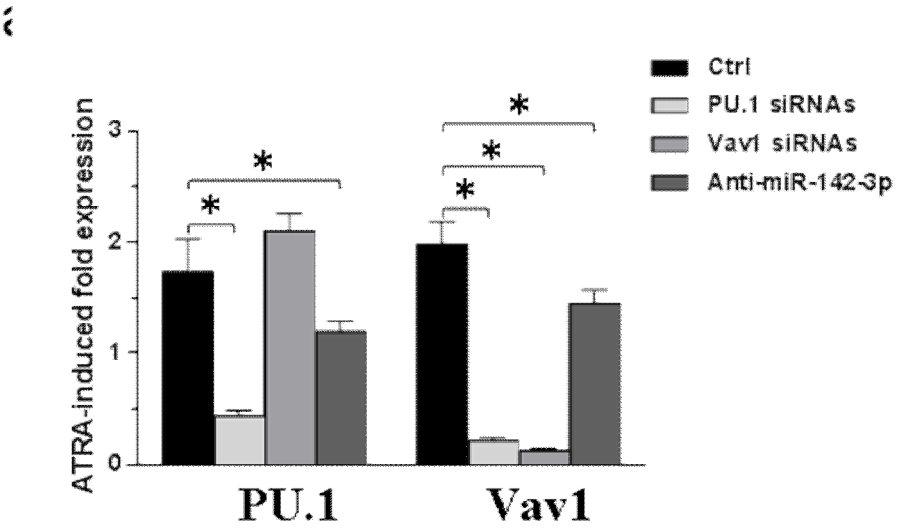

a
Ctrl anti-miR
Anti-miR-142-3p
0
0,5
1
1,5
Relative levels
b
Ctrl anti-miR
Ctrl anti-miR + ATRA
Anti-miR-142-3p + ATRA
c
Ctrl anti-miR
Ctrl anti-miR + ATRA
Anti-miR-142-3p + ATRA
d
CD11b expression (MFI)
350
300
250
200
150
100
50
0
*
Ctrl anti-miR
Ctrl anti-miR + ATRA
Anti-miR-142-3p + ATRA
e
Ctrl anti-miR + + -
ATRA - + +
Anti-miR-142-3p - - +
Vav1
PU.1
β-Tubulin
97 kDa
40 kDa
55 kDa

Figure 3


Ctrl
PU.1 siRNAs
Vav1 siRNAs
Anti-miR-142-3p
ATRA-induced fold expression
0
1
2
3
*
*
*
*
*
PU.1
Vav1

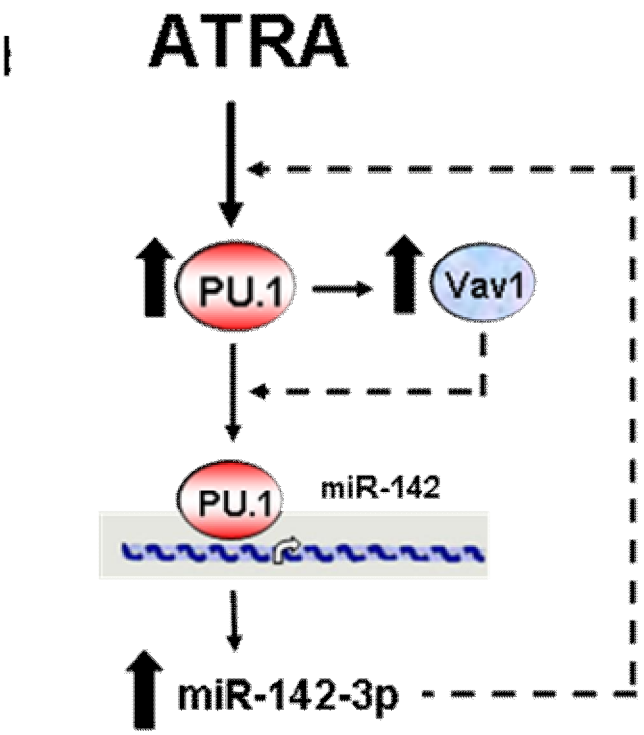

ATRA
PU.1
Vav1
PU.1
miR-142
miR-142-3p

Figure 4