

SHORT COMMUNICATION

A restricted signature of miRNAs distinguishes APL blasts from normal promyelocytesS Careccia^{1,8}, S Mainardi^{1,8,9}, A Pelosi¹, A Gurtner¹, D Diverio², R Riccioni³, U Testa³, E Pelosi³, G Piaggio¹, A Sacchi¹, S Lavorgna^{4,5}, F Lo-Coco^{4,5}, G Blandino^{1,6}, M Levrero^{6,7} and MG Rizzo¹¹Laboratory of Molecular Oncogenesis, Department of Experimental Oncology, Regina Elena Cancer Institute, Rome, Italy;²Department of Cellular Biotechnologies and Hematology, Sapienza University of Rome, Rome, Italy; ³Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy; ⁴Department of Biopathology, University of Rome 'Tor Vergata', Rome, Italy; ⁵Laboratorio di Neuro-Oncoematologia, Fondazione Santa Lucia, Rome, Italy; ⁶Rome Oncogenomic Center (ROC), Rome, Italy and ⁷Department of Internal Medicine, Sapienza University of Rome and Laboratory of Gene Expression, Fondazione Andrea Cesalpino, Rome, Italy

MicroRNAs (miRNAs) are small non-coding RNAs involved in the regulation of critical cell processes such as apoptosis, cell proliferation and differentiation. A small set of miRNAs is differentially expressed in hematopoietic cells and seemingly has an important role in granulopoiesis and lineage differentiation. In this study, we analysed, using a quantitative real-time PCR approach, the expression of 12 granulocytic differentiation signature miRNAs in a cohort of acute promyelocytic leukemia (APL) patients. We found nine miRNAs overexpressed and three miRNAs (miR-107, -342 and let-7c) downregulated in APL blasts as compared with normal promyelocytes differentiated *in vitro* from CD34+ progenitors. Patients successfully treated with all-trans-retinoic acid (ATRA) and chemotherapy showed downregulation of miR-181b and upregulation of miR-15b, -16, -107, -223, -342 and let-7c. We further investigated whether the APL-associated oncogene, promyelocytic leukemia gene (PML)/retinoic acid receptor α (RAR α), might be involved in the transcriptional repression of miR-107, -342 and let-7c. We found that PML/RAR α binds the regulatory sequences of the intragenic miR-342 and let-7c. In addition, we observed, in response to ATRA, the release of PML/RAR α paralleled by their transcriptional activation, together with their host genes, EVL and C21orf34 α . In conclusion, we show that a small subset of miRNAs is differentially expressed in APL and modulated by ATRA-based treatment. *Oncogene* advance online publication, 14 September 2009; doi:10.1038/onc.2009.255

Keywords: microRNAs; acute promyelocytic leukemia; PML/RAR α

Acute promyelocytic leukemia (APL) is a specific acute myelogenous leukemia subtype characterized by maturation arrest at the promyelocytic stage of development and caused by a novel fusion protein resulting from the reciprocal translocation involving the retinoic acid receptor α (RAR α) on chromosome 17 with the promyelocytic leukemia gene (PML) on chromosome 15. The resulting APL-associated PML/RAR oncogene (PML/RAR α) contributes to the maturation arrest of hematopoietic precursors and leukemogenesis by disrupting PML function and silencing the retinoic acid signaling pathway (Melnick and Licht, 1999; Tallman *et al.*, 2002).

MicroRNAs (miRNAs) are small non-protein-coding RNAs that regulate gene expression at the post-transcriptional level and influence many aspects of cell biology (Bartel, 2004; Meltzer, 2005; Pasquinelli *et al.*, 2005; Mallardo *et al.*, 2008). A number of miRNAs have been shown to be differentially expressed in hematopoietic tissues and to have an important role both in lineage differentiation and in human hematological malignancies (Chen *et al.*, 2004; Fazi *et al.*, 2005; Fontana *et al.*, 2007; Fabbri *et al.*, 2008). Among these, miRNA-223 is a key member of a regulatory circuitry involving C/EBP α and NFI-A that controls granulocytic differentiation in all-trans-retinoic acid (ATRA)-treated APL cells (Fazi *et al.*, 2005). More recently, it has been reported that treatment *in vitro* of the APL cell line NB4 with the differentiating agent ATRA leads to downregulation of miR-181 and upregulation of nine miRNAs, revealing a potential tumor suppressor function of these miRNAs in APL (Garzon *et al.*, 2007). In this study, we analysed the expression of 12 selected granulocytic signature miRNAs and the impact of ATRA-based therapy in a cohort of APL patients. We found that miR-15a, -15b, -16, 142-3p, -142-5p, -181b, -223, let-7a and let-7d are increased, whereas miR-107, -342 and let-7c are decreased in APL blasts obtained at the time of diagnosis as compared with human normal promyelocytes differentiated *in vitro* for 7 days from umbilical cord blood (UCB)-derived CD34+ hematopoietic progenitors (Figure 1).

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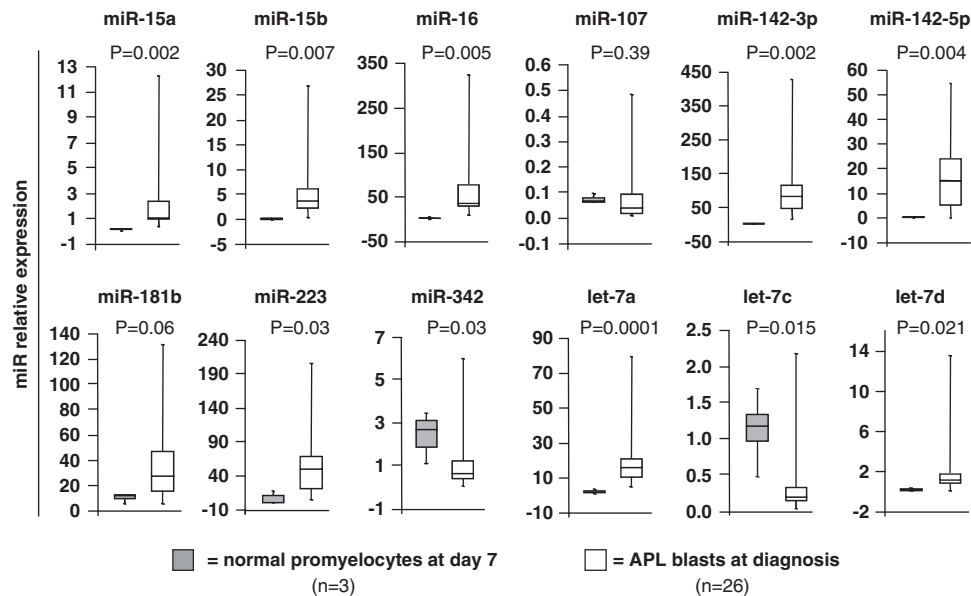


Figure 1 miRNA signatures in normal human promyelocytes and APL blasts. Box-plot diagrams of relative miRNAs expression levels in leukemic blasts (marrow samples with >90% of leukemic cell infiltration) from 26 APL patients at diagnosis versus miRNAs expression in normal promyelocytes derived from three independent experiments of normal granulocytic lineage differentiation. Boxes define the 25th and 75th percentiles; the horizontal line into the boxes indicates the median, and bars define the 10th and the 90th percentiles. Methods: total RNA was isolated using Trizol Reagent (Invitrogen). The indicated mature miRNAs were assayed by reverse transcriptase reaction using stem-loop primers followed by quantitative reverse transcriptase (qRT)-PCR performed in accordance with manufacturer's instructions (Applied Biosystems Inc., Foster City, CA, USA) on an Applied Biosystem 7500 Real Time PCR System SDS v1.2. Samples were normalized to RNU6B small RNA. Real-time PCR was performed in triplicate, including no-template controls. Relative expression was calculated using the comparative Ct method. *P*-values were determined using the Mann-Whitney rank sum test.

To gain further insights into the specificity of the miRNAs signature of APL blasts as compared with different stages of granulocytic differentiation, we compared the expression of our set of miRNAs in UCB-derived CD34⁺ unilineage granulocytic cultures at different stages of normal granulopoiesis. This serum-free *ex vivo* culture systems of granulocytic lineage differentiation allows the analysis of 'pure' unilineage precursors at discrete sequential stages of development, starting from initial UCB-derived CD34⁺ progenitor cells through terminal granulocyte maturation in a 2-week period, as indicated by cell morphology at days 0, 7 and 15 (Figures 2a and b), and by increased expression of granulocytic-specific membrane antigens (data not showed). As shown in Figure 2c, we found a strong upregulation (fold >5) of miR-15a, -15b, -107, -181b, -223, let-7a, let-7d and to a lesser extent (2 < fold < 5) of miR-16, -142-3p, -142-5p and miR-342 in promyelocytes as compared with that in UCB-derived CD34⁺ cells. We also found that high levels of miR-15b, -223, -142-5p and -342 (fold >2) and reduced expression of miR-181b and let-7c distinguished fully differentiated granulocytes from normal promyelocytes. Altogether these findings define a specific miRNA signature of APL blasts at diagnosis that differs from normal promyelocytes.

Next, we evaluated the impact of a standard ATRA-containing regimen on selected miRNAs in leukocyte APL patients obtained at time of remission (after third

consolidation). We found that most successfully treated patients displayed a striking decrease of miR-181b expression (91.7% of patients), whereas in the majority of patients miR-15b (83.3%), -16 (70.8%), -107 (87.5%), -223 (91.7%), -342 (83.3%) and let-7c (62.5%) were significantly increased after therapy (Figures 3a and b). Interestingly, whereas upregulation of miR-15b and -342 in treated patients mimics the behavior of these miRNAs in the differentiation of normal promyelocytes toward mature granulocytes, upregulation of miR-16 and -107 (that are not upregulated in granulocytes as compared with those in promyelocytes) and let-7c (that decreases all along granulocytic differentiation) is likely related to a specific effect of ATRA. This conclusion is further supported by the observation that, in contrast to the parental NB4 cells, the ATRA-resistant NB4 sub-clone MR2 cells (Rosenauer *et al.*, 1996), showed no significant modulation in the selected miRNA expression in response to ATRA, with the exception of miR-16, that was downregulated, and miR-223 that was moderately upregulated (Figure 3c). We also analysed the expression levels of the same set of differentiation-related miRNAs in sequential samples obtained from three patients (#46, #48, #50) who underwent disease relapse during follow-up. All patients showed a strong upregulation of miR-15b, -107, -223, -342, let-7c and, to a lesser extent, of miR-15a and miR-16 in samples collected during remission. In patients #46 and #50, who underwent

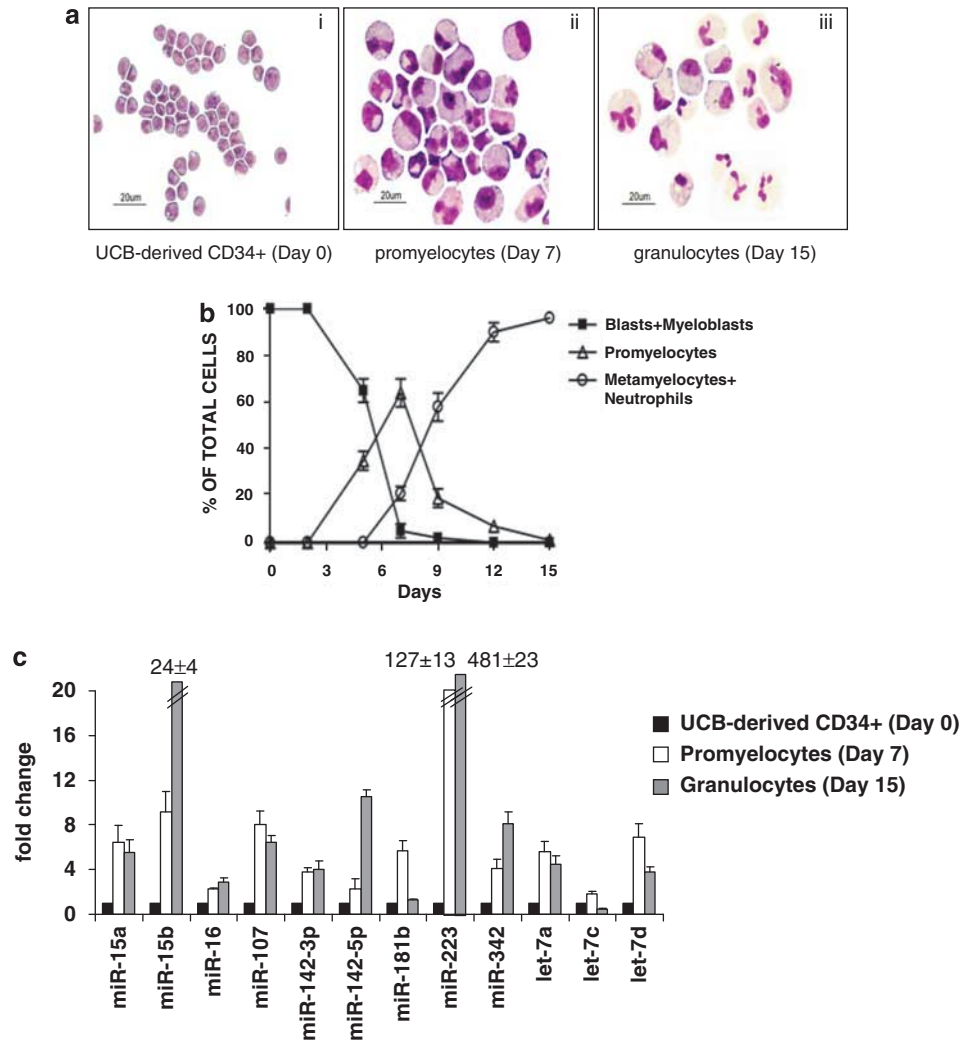


Figure 2 Changes in the expression levels of differentiation-related microRNAs during serum-free *ex vivo* granulocytic differentiation. (a) May-Grünwald-Giemsa staining at sequential stages of UCB-derived CD34+ progenitors induced to differentiate along the granulocytic lineage. (b) Kinetic changes in the percentages of blasts + myeloblasts, promyelocytes and metamyelocytes + neutrophils at different days during normal granulocytic lineage differentiation. (c) qRT-PCR of the 12 selected miRNAs in UCB-derived CD34+ cells, promyelocytes and granulocytes. Results are expressed as fold changes (mean \pm s.d. from three independent experiments) in miRNAs expression in promyelocytes and terminally differentiated granulocytes as compared with the values observed in UCB-derived CD34+ cells. Methods: serum-free *ex vivo* cultures induced to differentiate along the granulocytic lineage in the presence of a growth factor-defined medium were obtained as previously described (Testa *et al.*, 1996). RNA extraction, reverse transcriptase (RT)-PCR reactions and mature miRNAs quantification were performed as described in the legend of Figure 1.

hematological relapse, all these miRNA were sharply downregulated at time of disease recurrence, returning to levels comparable to or lower than those observed at diagnosis (Figure 3d). In patient #48, who had a molecular relapse at the time of its second testing, we observed a sharp downregulation of miR-15b and -223, but not of miR-107, -342 and let-7c (Figure 3d). Whether the difference observed between patients with overt relapse and early molecular relapse are due to true biological differences, or are simply related to the timing of analysis or reflect the dilution of the re-emerging leukemic clone, will require further investigations.

Altogether, these results expand and confirm *in vivo* previous observations obtained *in vitro* (Garzon *et al.*,

2007; Saumet *et al.*, 2009) and suggest a role of these miRNAs in therapeutic response in APL patients.

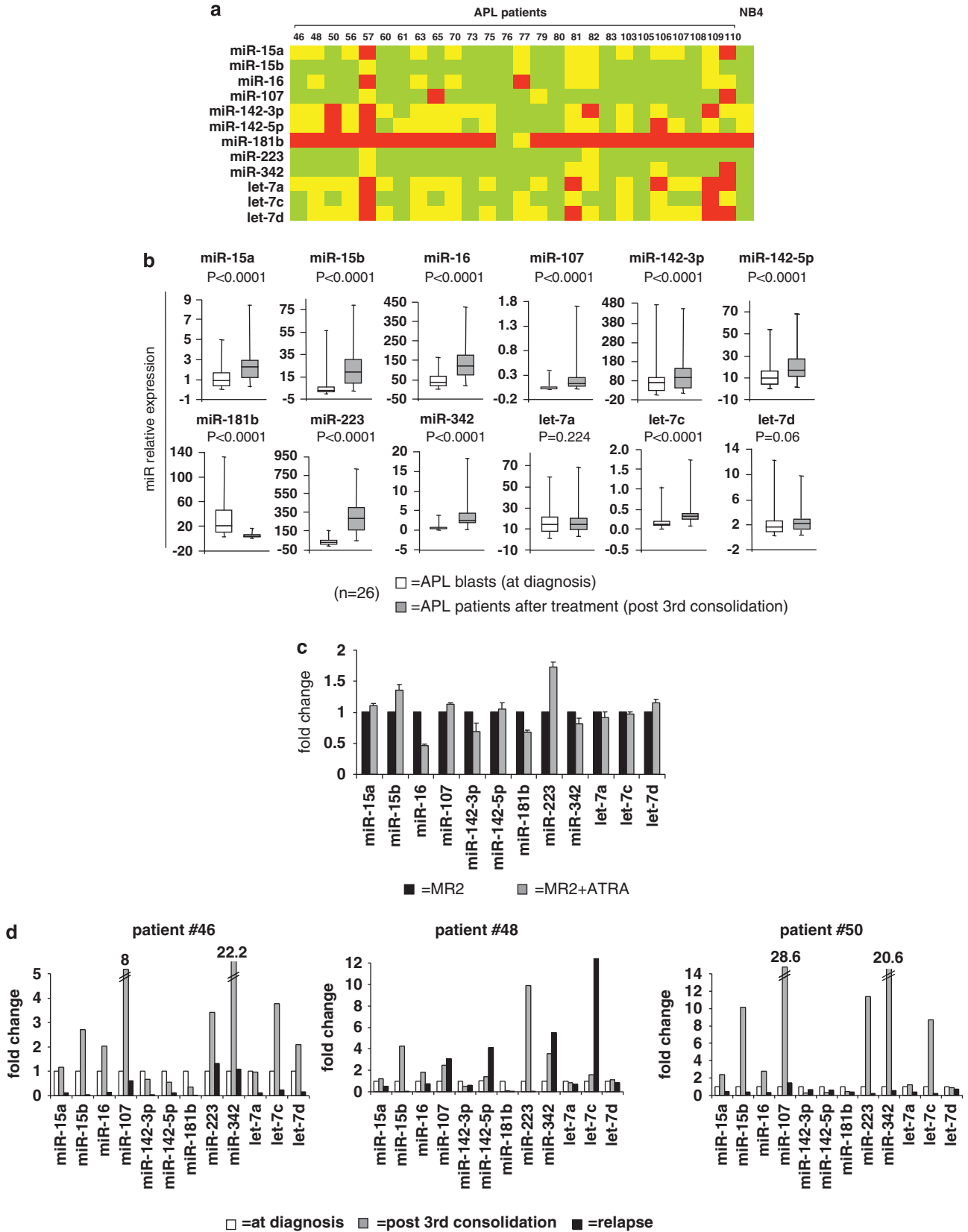
We then focused on the regulation of miR-107, -342 and let-7c. These miRNAs were all decreased in APL blasts as compared with those in normal promyelocytes (Figure 1), and strikingly increased in APL patients after ATRA-based therapy (Figures 3a and b).

miR-107, -342 and let-7c are intragenic; they are located in the introns of protein-encoding genes and are supposedly transcribed coincidentally with their host genes (Rodriguez *et al.*, 2004; Kim and Kim, 2007; Grady *et al.*, 2008).

First, we confirmed that miR-107, -342 and let-7c are co-regulated with their respective host genes PANK1, EVL and C21orf34 α in promyelocytic NB4 cells exposed for

120 h to ATRA (Figure 4a, left panel). Moreover, *in silico* analysis of the putative PANK1/miR-107, EVL/miR-342 and C21orf34 α /let-7c promoters revealed the presence

of multiple RARE sites (Figure 4c). In the case of let-7c an additional RARE site was found in the C21orf34a intron 6, upstream of the let-7c initiation site (Figure 4c,



lower panel). To investigate the role of PML/RAR α in ATRA-mediated regulation of miR-107, -342 and let-7c expression, we performed experiments in PR9 cells, a U937-derived cell line in which exogenous PML/RAR α expression is Zinc-inducible (Grignani *et al.*, 1993). As shown in Figure 4b (right panel), zinc-induced expression of PML/RAR α led to a clear-cut decrease of miR-342 and let-7c transcripts, whereas ATRA treatment strongly induced their expression. MiR-107 expression was not affected by PML/RAR α induction, suggesting that in the case of miR-107, induction by ATRA might be due to an indirect mechanism.

These experiments prompted us to investigate whether the ability of ATRA treatment to induce miR-107, -342 and let-7c expression might be mediated in NB4 cells by a release of PANK1, EVL and C21orf34 α promoters from endogenous PML/RAR α repression. Using chromatin immunoprecipitation, we found that PML/RAR α binds *in vivo* with variable affinity to the four RARE sites located in the promoter regions of EVL/miR-342 and two out of three RARE sites of the C21orf34 α /let-7c promoter, but not the C21orf34 α intronic RARE sites and the RARE sites in the PANK1 promoter (Figure 4d). As expected, the lack of PML/RAR α recruitment onto the PANK1/miR-107 promoter confirms the results obtained in PR9 cells (Figure 4b, right panel) and strongly suggests that miR-107 modulation by ATRA is PML/RAR α -independent and might be due to indirect mechanisms. After ATRA treatment, PML/RAR α was released from all the occupied RARE sites (Figure 4d) and this correlated with the recruitment of the acetyltransferase p300 and an increased H3 histone acetylation (PAN-H3ac, H3K9ac and H3K14ac) around the RARE elements of both EVL/miR-342 and C21orf34 α /let-7c promoters together with a sharp decrease in lysine 9 trimethyl-histone H3 (H3K9me3) at the RARE site proximal to the C21orf34 α transcription start site (Figure 4d). Of note, when similar chromatin immunoprecipitation experiments were performed in the ATRA-resistant MR2 cells, we found that PML/RAR α was retained on EVL/miR-342 and C21orf34 α /let-7c promoters and that, accordingly, histone

H3 acetylation status was not modified after ATRA treatment (Figure 4e).

These results confirm that the EVL/miR-342 and C21orf34 α /let-7c promoters are targeted by PML/RAR α in APL cells. Although additional mechanisms may be at work (that is, mutations of the PML/RAR α fusion protein or deletion-specific miR loci modulated by ATRA), our observations strongly suggest that the ATRA-mediated changes of miR-342 and let-7c transcription are a specific effect of ATRA treatment in APL patients *in vivo*.

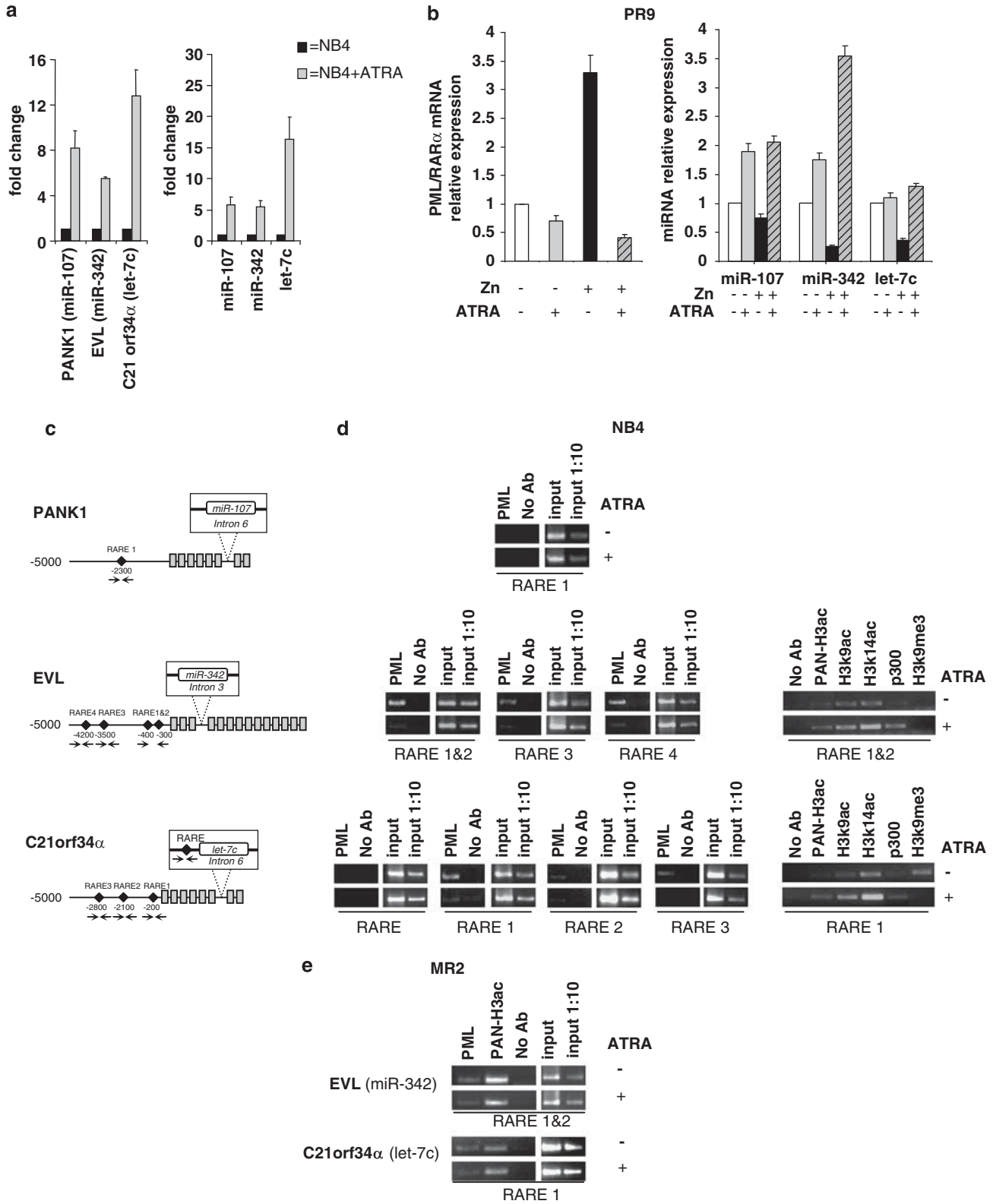
The miRNA target prediction analysis performed using Pictar, miRanda, TargetScan and miRBase Targets software has confirmed a number of putative targets for miR-107, -342 and let-7 family that might have a role in APL pathogenesis and/or ATRA treatment response. miR-107 has been shown to target the transcription factor NFI-A (Garzon *et al.*, 2007), which participates with C/EBP α in the regulation of granulocyte differentiation-associated miR-223 expression (Fazi *et al.*, 2005). miR-342 has been shown to stimulate granulocytic differentiation (De Marchis *et al.*, 2009). Interestingly, the putative target of miR-342 MEIS1, a member of the TALE family of homeodomain genes, has been shown to have a pivotal role in normal hematopoiesis (Abramovich and Humphries, 2005; Diaz-Blanco *et al.*, 2007) and it is frequently upregulated in human AMLs (Kumar *et al.*, 2009). Finally, it is known the involvement of let-7 family members in differentiation and development, as well as in anti-proliferative functions, by targeting the RAS oncogene and the non-histone DNA binding protein HMG A2 (Johnson *et al.*, 2005; Peng *et al.*, 2008).

In conclusion, we identified a small subset of miRNAs that are modulated during normal granulocyte differentiation and represent new markers in the therapeutic response in APL patients.

Conflict of interest

The authors declare no conflict of interest.

Figure 3 Effects of ATRA treatment *in vivo* on the expression levels of differentiation-related microRNAs in APL patients. (a) Heat-map of miRNA expression, analysed by qRT-PCR, in APL patient samples after treatments (after third consolidation) with respect to the samples at diagnosis and in ATRA-treated with respect to untreated NB4 cells. Green boxes: increased miRNAs (fold > 2); red boxes: decreased miRNAs (> 50%), yellow boxes: no significantly change. (b) Box-plot diagrams of miRNAs expression at the time of diagnosis and after completion of the treatment protocol. Boxes define the 25th and 75th percentiles; the horizontal line into the boxes indicates the median, and bars define the 10th and the 90th percentiles. *P*-values were determined with the paired Wilcoxon's test. (c) Expression levels of the 12 selected miRNAs in untreated and ATRA-treated (2 μ M, 120h) NB4 sub-clone, MR2. Results (mean \pm s.d. from three independent experiments) are expressed as fold changes in ATRA-treated relative to untreated MR2 cells. (d) qRT-PCR of 12 selected miRNAs in bone marrow sample from APL patients #46, #48 and #50 analysed also at the time of clinical relapse. Results are expressed as fold changes at the end of the treatment protocol (after third consolidation) and at the time of clinical relapse relative to the time of diagnosis. Methods: Bone marrow specimens of APL patients enrolled in the ATRA plus IDArubicin (AIDA) protocol of the Italian Cooperative Group GIMEMA (Mandelli *et al.*, 1997) were collected in all cases at the time of diagnosis and after completion of the treatment protocol (induction followed by three courses of consolidation). Informed consent was obtained from the patients or their parents, and the study was approved by the Institutional review board of the Department of Human Biotechnology and Hematology of University 'La Sapienza' of Rome. Main clinical characteristics of the APL patients are in Supplementary Table S1. RNA extraction, reverse transcriptase (RT)-PCR reactions and mature miRNAs quantification were performed as in the legend of Figure 1. Patients and MR2 samples were normalized using RNU19.



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Figure 4 PML/RAR α modulates transcription of the intragenic miR-342 and let-7c in acute promyelocytic leukemia (APL) cell lines. (a) mRNA expression levels of the host genes PANK1, EVL and C21orf34 α (Left), and of intragenic miR-107, -342 and let-7c (right) in untreated and ATRA-treated (2 μ M, 120 h) NB4 cells. (b) PML/RAR α (left panel), and miR-107, -342 and let-7c (right panel) expression levels in non-induced PR9 cells (PML/RAR α -negative), Zn-induced PR9 cells (induced PML/RAR α expression), either untreated or treated with ATRA. (c) Schematic representation of 5000 bp upstream regulatory regions of the host genes PANK1 (miR-107), EVL (miR-342) and C21orf34 α (let-7c). For C21orf34 α , a RARE site in intron 6, ~600 bp upstream from the let-7c initiation site is also shown. Arrows outline the promoter regions amplified by the specific primer pairs used for PCR amplification of immunoprecipitated chromatin. (d) Chromatin immunoprecipitation (ChIP) analysis of PML-RAR α occupancy, p300 and H3 histone modifications of the indicated host gene promoter regions in untreated and ATRA-treated NB4. (e) ChIP analysis of PML/RAR α occupancy and H3 histone acetylation in ATRA-resistant MR2 cells. Methods: RNA quantification of host genes PANK1, EVL and C21orf34 α in NB4 ATRA-treated (2 μ M, 120 h) or untreated cells was performed using SYBR Green DNA Master mix and specific primers. GAPDH gene expression was used as endogenous control. Results (mean \pm s.d. from three independent experiments) are expressed as fold changes in ATRA-treated relative to untreated cells. MicroRNAs quantification were performed as described in the Figure 1 using RNU19 as endogenous control. Results (mean \pm s.d. from three independent experiments) are expressed as fold changes in ATRA-treated relative to untreated NB4 and PR9 cells. PML/RAR α , cDNAs were amplified by qRT-PCR using specific primer pairs and TaqMan probe sets. ABL mRNA transcript was used as control to correct for RNA quality differences. Cross-linked chromatin from NB4 and MR2 cells were immunoprecipitated with the indicated antibodies and DNA amplified using specific primers as described (Mainardi *et al.*, 2007).

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>)