OPEN

Citation: Cell Death and Disease (2017) 8, e2849; doi:10.1038/cddis.2017.253
Official journal of the Cell Death Differentiation Association

www.nature.com/cddis

Knockdown of *miR-128a* induces *Lin28a* expression and reverts myeloid differentiation blockage in acute myeloid leukemia

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Lin28A is a highly conserved RNA-binding protein that concurs to control the balance between stemness and differentiation in several tissue lineages. Here, we report the role of *miR-128a/Lin28A* axis in blocking cell differentiation in acute myeloid leukemia (AML), a genetically heterogeneous disease characterized by abnormally controlled proliferation of myeloid progenitor cells accompanied by partial or total inability to undergo terminal differentiation. First, we found Lin28A underexpressed in blast cells from AML patients and AML cell lines as compared with CD34+ normal precursors. *In vitro* transfection of Lin28A in NPM1-mutated OCI-AML3 cell line significantly triggered cell-cycle arrest and myeloid differentiation, with increased expression of macrophage associate genes (*EGR2*, *ZFP36* and *ANXA1*). Furthermore, *miR-128a*, a negative regulator of *Lin28A*, was found overexpressed in AML cells compared with normal precursors, especially in acute promyelocytic leukemia (APL) and in 'AML with maturation' (according to 2016 WHO classification of myeloid neoplasms and acute leukemia). Its forced overexpression by lentiviral infection in OCI-AML3 downregulated Lin28A with ensuing repression of macrophage-oriented differentiation. Finally, knockdown of *miR-128a* in OCI-AML3 and in APL/AML leukemic cells (by transfection and lentiviral infection, respectively) induced myeloid cell differentiation and increased expression of *Lin28A*, *EGR2*, *ZFP36* and *ANXA1*, reverting myeloid differentiation blockage. In conclusion, our findings revealed a new mechanism for AML differentiation blockage, suggesting new strategies for AML therapy based upon *miR-128a* inhibition.

Cell Death and Disease (2017) **8,** e2849; doi:10.1038/cddis.2017.253; published online 1 June 2017

Acute myeloid leukemia (AML) is a heterogeneous hematopoietic stem cell neoplasm, characterized by rapid growth and/or impaired differentiation of leukemic cells with abnormal accumulation.[1–3] Recurring chromosomal aberrations and gene mutations contribute to AML pathogenesis and are the most important tools for classification and prognosis assessment of AML.[2–4] Furthermore, there are some known deregulated pathways involved in the maintenance of leukemic stem cells such as hedgehog,[5,6] tyrosine kinase receptors (e.g. Flt3),[3,7] Wnt and Notch.[8–11] Notwithstanding, a successful target therapy is not yet available. Improving our current knowledge on the biology of AML-associated leukemic processes represents a valuable tool to identify novel potential drug targets.

Lin28 is a conserved RNA-binding protein having an important role in cancer stem cells.[12,13] This protein is expressed in embryonic stem cells[14,15] and is capable, with OCT4, SOX2 and NANOG, of converting fibroblasts in induced pluripotent stem cells.[16] Lin28, by physical interaction with several RNA transcripts, exerts various forms of regulation ranging from alternative splicing, turnover, localization and translation.[17–19] It has been demonstrated that altered functionality of RNA-binding proteins, due to deregulated gene expression or gene mutations, often results in genetic disease and cancer.[20]

Several studies reported the existence of regulatory pathways between Lin28 and different miRNAs.[15,21–23] In murine model, overexpression of *miR-125b* leads to the downregulation of Lin28A and the preleukemic state characterized by overproduction of myeloid cells eventually progressing to a myeloid leukemia.[24–26] Conversely, ectopic expression of Lin28B reprograms hematopoietic progenitor cells from adult bone marrow (BM), endowing them to mediate multilineage reconstitution.[27] Moreover, Li *et al.*[22] showed that *miR-181* promotes megakaryocytic differentiation repressing Lin28 and upregulating let-7 expression. Thus, Lin28 seems to be a pivotal regulator of hematopoiesis. Interestingly, Lin28 is also regulated by *miR-128*,[28] a microRNA able to hold hematopoietic cells in an early progenitor stage, blocking their

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Received 14.2.17; revised 05.5.17; accepted 05.5.17; Edited by M Diederich

differentiation towards more mature cells.[29,30] Moreover, this microRNA was found associated with AML.[31–33] Therefore, it will be appealing to gain further insights into the role of *miR-128a/Lin28A* axis in induction and maintenance of an early differentiation status in AML.

Results

***Lin28A* expression was downregulated in myeloid leukemic cells.** To evaluate *Lin28A* expression in AML, we performed quantitative real-time-PCR (qRT-PCR) in isolated blast cell samples from 38 AML patients at diagnosis, 7 AML cell lines (OCI-AML3, KG-1, Kasumi-1, NB4, CMK, ME-1 and MOLM-14) and CD34+ purified samples from 13 healthy donors. *Lin28A* ($P<0.01$) and cell lines (OCI-AML3 and KG-1 $P<0.001$, Kasumi-1, NB4, CMK and ME-1, $P<0.01$) showed a significantly lower expression in AML patients as compared with controls (Figure 1a). To support our data, we also analyzed two independent publicly available gene expression profiling data sets, one containing 16 CD34+ isolated samples from healthy subjects (GSE 42519), and one with 251 AML patients with newly diagnosed AML (GSE 15434) confirming a significant downregulation of *Lin28A* in AML patients (230 BM and 21 PB) compared with healthy subjects (Supplementary Figure 1a). Stratifying AML according to the WHO classification,[4] *Lin28A* value was found underexpressed in all AML subtypes (Figure 1b) compared with controls. Stratifying AML cases according to the principal genomic alterations detected in our cohort of patients and in GSE 15434 data set, we found lower expression of Lin28A in AML patients independent of their specific alterations (Figure 1c and Supplementary Figure 1b). Moreover, we evaluated Lin28A protein by cytometric analysis detecting a lower percentage of Lin28A+ cells in AML blast cells compared with normal hematopoietic myeloid precursors ($P<0.01$) (Figure 1d). When we analyzed distinct subsets of normal CD34+ cells, we observed a higher percentage of Lin28A+ cells in normal myeloid precursors (CD33+) compared with the erythroid (CD71+) ($P<0.01$) and lymphoid (CD19+) ($P<0.001$) ones, suggesting its main involvement in myeloid differentiation (Figure 1e).

Lin28A overexpression induced hematopoietic differentiation in AML. To examine the effect of Lin28A in AML, we transfected OCI-AML3 cells with Lin28A plasmid. The significant increase of Lin28A protein expression was confirmed by western blot and cytofluorimetric analysis ($P<0.01$ at 24 h and $P<0.05$ at 48 h, in both cases) (Figures 2a–d). Lin28A overexpression was associated with the induction of monocyte/macrophage-like differentiation. In fact, flow cytometric analysis revealed a higher percentage of CD11b – ($P<0.05$ at 48 h) and CD14+ cells ($P<0.01$ at 24 h and $P<0.001$ at 48 h) after Lin28A transfection (Figures 2e–h). Ectopic expression of Lin28A also significantly increased p21 protein levels ($P<0.001$ at 24 h and $P<0.05$ at 48 h), inducing cell-cycle arrest in the S phase ($P<0.01$ at 24 h and $P<0.001$ at 48 h) (Figures 2a and i). Consistent to the ability of Lin28A in inducing hematopoietic differentiation in AML cells, we detected a significant increase of *EGR2* and *ZFP36*, two key regulators of monocyte/macrophage differentiation (Figures 2j–k),[34–36] and *ANXA1*, a gene normally stored in inside macrophage cytosol (Figure 2l)[37] after Lin28A overexpression at 24–48 h.

***Lin28A* expression increased during PMA or ATRA differentiation.** To corroborate the involvement of Lin28A in myeloid differentiation, we stimulate AML cell lines to differentiate. In particular, we induced macrophage-like differentiation treating ME-1/OCI-AML3 cell lines with phorbol 12-myristate 13-acetate (PMA) and MOLM-14 with all-*trans*-retinoic acid (ATRA), and granulocyte-like differentiation treating NB4 and KG-1 cell lines with ATRA. After treatment, the cytometric data revealed a significant percent increase, from 24 to 72 h, of CD11b+ cells and CD14+ cells in ME-1, OCI-AML3 (Figures 3a and b) and NB4 (Supplementary Figure 2a), of CD11b and CD11c in MOLM-14 (Figure 3c) and of CD11b and CD15 in KG-1 (Supplementary Figure 2b). To confirm cytometric analysis of cell differentiation, we detected by qRT-PCR a significant augment, in all time points, of *EGR2*, *ZFP36* and *ANXA1* in treated ME-1, OCI-AML3, MOLM-14 (Figures 3d–f) and NB4 (Supplementary Figure 2c).[37] As expected, at the same time, we observed a significant upregulation of Lin28A and an increased percentage of Lin28A+ cells in all cell lines (Figures 3g–j and Supplementary Figures 2d and e). Similarly to Lin28A transfection, PMA and ATRA treatment of AML cell lines also induced p21 expression (Figures 3g–i) and a significant cell-cycle arrest in the G2 phase (ME-1: $P<0.001$ at 48 h, $P<0.05$ at 72 h; OCI-AML3: $P<0.01$ at 24 h, $P<0.05$ at 48 and 72 h; KG-1: $P<0.001$ at 24 h, $P<0.05$ at 48 h, $P<0.001$ at 72 h) (Figure 3k and Supplementary Figure 2f), the G1 phase (MOLM-14: $P<0.001$ at 72 h) (Figure 3k) or the S phase (NB4: $P<0.001$ at 24 h, $P<0.01$ at 48 and 72 h) (Supplementary Figure 2f).

***MiR-128a* expression was upregulated in myeloid leukemic cells.** To further clarify Lin28A downregulation in AML, we analyzed its regulator, *miR-128a*.[28] We evaluated *miR-128a* expression in the same cohort of AML patients and in the AML cell line panel previously examined for *Lin28A*, observing a significant overexpression of this microRNA compared with healthy subjects (Figure 4a). Stratifying AML cases for morphologic features, we found, at variance with Lin28A, elevated expression levels of *miR-128a* in AML with maturation and acute promyelocytic leukemia (APL) cases compared with controls (Figure 4b). Furthermore, considering patients for their gene mutations, we found a significantly higher expression of *miR-128a* in patients with FLT3, PML/RARα and other genomic alterations (Figure 4c).

Our results show different expression pattern of *miR-128a* in MOLM-14 and AML samples, both carrying FLT3-ITD (Figures 4a and c). Matsuo *et al*.[38] demonstrated that MOLM-14, along with FLT3-ITD, carries a series of genotypic aberrancies, such as the insertion ins(11;9) with the fusion hybrid MLL-AF9.[38] This complex pattern could justify the partially divergent behavior of MOLM-14 as compared with fresh AML samples. Moreover, we also evaluated, by qRT-PCR, *miR-128a* expression during macrophage- and granulocytic-like differentiation detecting a significant

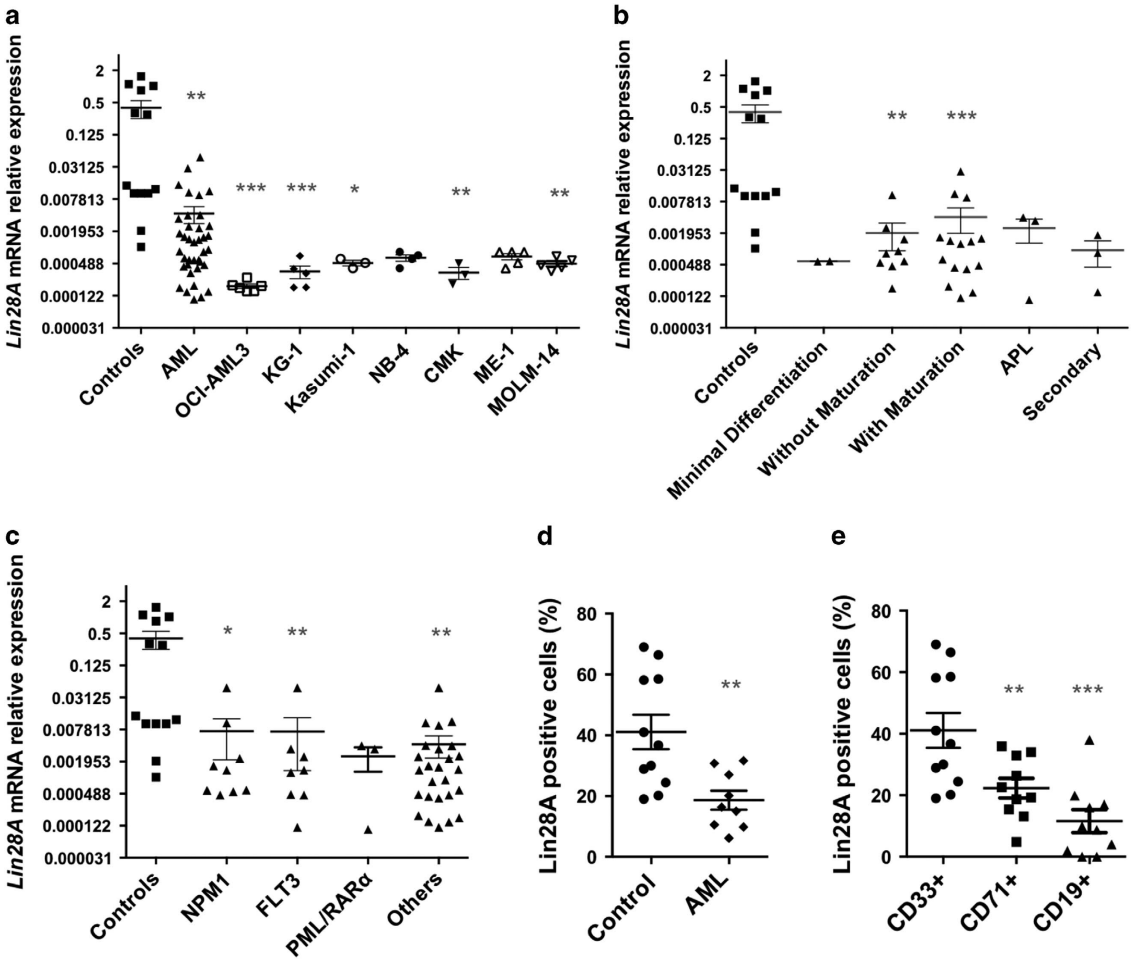

Figure 1 *Lin28A* expression in leukemic blasts from AML patients. (**a**) qRT-PCR of *Lin28A* in 13 healthy controls, 38 AML patients and 7 AML cell lines (OCI-AML3, KG-1, Kasumi-1, NB4, CMK, ME-1 and MOLM-14); *ABL1* was used for normalization. Relative values were calculated on the basis of the ΔCp method. Results are shown as mean ± S.E.M. (**b**) Expression of *Lin28A* mRNA in AML patients stratified for morphologic features (with minimal differentiation, $n=2$; without maturation, $n=9$; with maturation including: $n=3$ with maturation, $n=10$ acute myelomonocytic leukemia, $n=2$ acute monoblastic/monocytic leukemia; APL, $n=3$; secondary AML, $n=3$) was compared with 13 healthy controls. Results are shown as mean ± S.E.M. (**c**) Expression of *Lin28A* mRNA in AML patients with specific mutations (NPM1, $n=9$; FLT3, $n=8$; PML/RARα, $n=3$ or with other alterations, $n=26$) was compared with 13 healthy controls. Results are shown as mean ± S.E.M. (**d**) Percentage of Lin28A+ cells in 11 BM healthy controls and 9 AML patients, by cytofluorimetric analysis. (**e**) Percentage of Lin28A+ cells in normal myeloid (CD34+ CD45+ CD33+), erythroid (CD34+ CD45+ CD71+) and lymphoid (CD34+ CD45+ CD19+) precursors, by cytofluorimetric analysis. Statistically significant analyses are indicated by asterisks: *$P<0.05$, **$P<0.01$ and ***$P<0.001$

downregulation of this *microRNA* from 24 to 72 h in treated cell lines (Figure 4d and Supplementary Figure 3a). To determine the role of *miR-128a* in myeloid differentiation, we transiently transfected OCI-AML3 and ME-1 cells with anti-*miR-128a.* After transfection, the inhibition of *miR-128a* (Figure 4e and Supplementary Figure 4a) and the increase of *ZFP36* were confirmed by qRT-PCR assay (Figure 4f and Supplementary Figure 4b), thus supporting a role of *miR-128a* in monocyte/macrophage differentiation. Furthermore, to confirm a *miR-128/Lin28A* axis, we evaluated *Lin28A* expression after anti-*miR-128a* transfection, confirming its upregulation in both cell lines (Figure 4g and Supplementary Figure 4c); we also observed an increase of p21 in OCI-AML3 cells (Figure 4g).

***MiR-128a* overexpression altered macrophage- and granulocytic-like differentiation.** To examine the effect of *miR-128a* in AML, we overexpressed by lentiviral infection its microRNA precursor (pLKO.1_*miR-128a*) and, as a control, an empty vector (pLKO.1_*scr*) in OCI-AML3 (Supplementary Figure 5). After lentivirus infection, cells were treated with PMA to differentiate in macrophage-like cells. Although during differentiation *miR-128a* expression seemed to be reduced in treated cells, its levels remained significantly higher in pLKO.1_*miR-128a* cells than in pLKO.1_*scr* cells ($P<0.05$ at 24 h, $P<0.01$ at 48 h and 72 h) (Figure 5a). Concurrently, *Lin28A* expression increased as a consequence of the induction culture, but it was significantly downregulated in OCI-AML3 infected with *miR-128a* ($P<0.05$ at 24–72 h) compared with control (Figure 5b).

Overexpression of *miR-128a* inhibited macrophage-like differentiation markers. In fact, flow cytofluorimetric data showed a reduction of CD11b+ and CD14+ cells after 24, 48 and 72 h of treatment with PMA (Figures 5c–e) in pLKO.1_*miR-128a* cells compared with that in pLKO.1_*scr* cells ($P<0.05$ at 72 h). These data were confirmed by morphologic analysis with May–Grünwald Giemsa staining

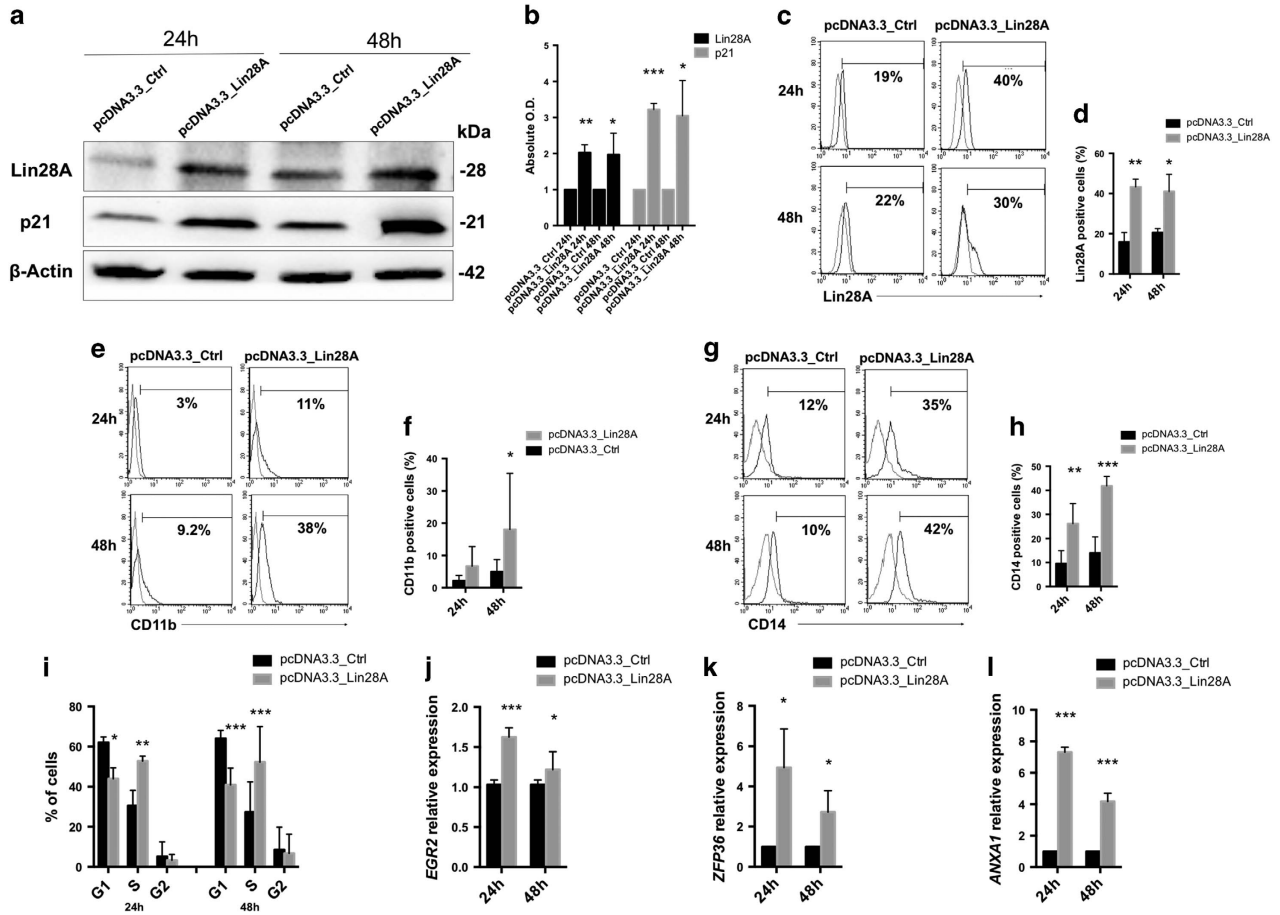

Figure 2 Overexpression of Lin28A in OCI-AML3 cell line. (**a**) Western blotting (WB) analysis of Lin28A, p21 and β-actin in OCI-AML3 after 24 and 48 h of transfection with pcDNA3.3_Ctrl or pcDNA3.3_Lin28A plasmids. (**b**) Absolute OD values of (**a**) were normalized to β-actin and shown as mean $\pm$ S.D. from two independent experiments. (**c**) Representative cytofluorimetric analysis of percentage Lin28A+ cells in OCI-AML3 after 24 and 48 h of transfection with pcDNA3.3_Ctrl or pcDNA3.3_Lin28A plasmids. (**d**) Percentage of Lin28A+ OCI-AML3 cells after 24 and 48 h of transfection with pcDNA3.3_Ctrl or pcDNA3.3_Lin28A plasmids, by cytofluorimetric analysis. (**e**) Representative cytofluorimetric analysis of CD11b+ cells in OCI-AML3 after 24 and 48 h of transfection with pcDNA3.3_Ctrl or pcDNA3.3_Lin28A plasmids. (**f**) Percentage of CD11b+ OCI-AML3 cells after 24 and 48 h of transfection with pcDNA3.3_Ctrl or pcDNA3.3_Lin28A plasmids. (**g**) Representative cytofluorimetric analysis of CD14+ cells in OCI-AML3 after 24 and 48 h of transfection with pcDNA3.3_Ctrl or pcDNA3.3_Lin28A plasmids. (**h**) Percentage of CD14+ OCI-AML3 cells after 24 and 48 h of transfection with pcDNA3.3_Ctrl or pcDNA3.3_Lin28A plasmids, by cytofluorimetric analysis. (**i**) Cell-cycle analysis in OCI-AML3 after 24 and 48 h of transfection with pcDNA3.3_Ctrl or pcDNA3.3_Lin28A plasmids. (**j–l**) qRT-PCR of *EGR2* (**j**) *ZFP36* (**k**) and *ANXA1* (**l**) in OCI-AML3 after 24 and 48 h of transfection with pcDNA3.3_Ctrl or pcDNA3.3_Lin28A plasmids. The bar graphs represented mean $\pm$ S.D. from three independent experiments. Statistically significant analyses are indicated by asterisks: $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$

of infected cells, highlighting that *miR-128a* overexpression led to less mature macrophage-like cells (Figure 5f). Moreover, lentiviral infection of *miR-128a* inhibited colony-forming activity of colony-forming unit-macrophage (CFU-M) in colony size and number (Figures 5g and h).

Inhibition of *miR-128a* improved myeloid differentiation in AML BM HSPC. Since significantly increased *miR-128a* expression was mainly observed in AML with maturation, we investigated how *miR-128a* inhibition could influence myeloid differentiation/maturation blockage. Lenti-miRZip-128a stably expresses hairpins that have anti-miRNA activity. We used BM HSPCs derived from two AML patients with maturation (myeloblastic AML3 and myelomonocyte AML2, respectively), both FLT3 mutated, and one APL patient (AML1) (Supplementary Table S1). BM HSPCs were infected with Lenti-miRZip-128a or Lenti-GFP and exposed to macrophage-like induction culture. Flow cytometric analysis showed a significant increased of CD11b and CD14 percentage of positive cells in AML HSPCs infected with Lenti-miRZip-128a compared with the control (Figures 6a and b). Lenti-miRZip-128a infection decreased the levels of mature *miR-128a* (Figure 6c) and significantly enhanced the expression of *Lin28A*, *EGR2*, *ZFP36* and *ANXA1* (Figure 6d). These results demonstrated that *miR-128a* inhibition in AML induce myeloid differentiation.

Discussion

AMLs are clonal diseases of hematopoietic progenitor cells, characterized by marked heterogeneity in terms of phenotypic, genotypic and clinical features.[1,2,4,39] In this study, we showed that *Lin28A*, an RNA-binding protein,[12] was significantly underexpressed in AML samples without any

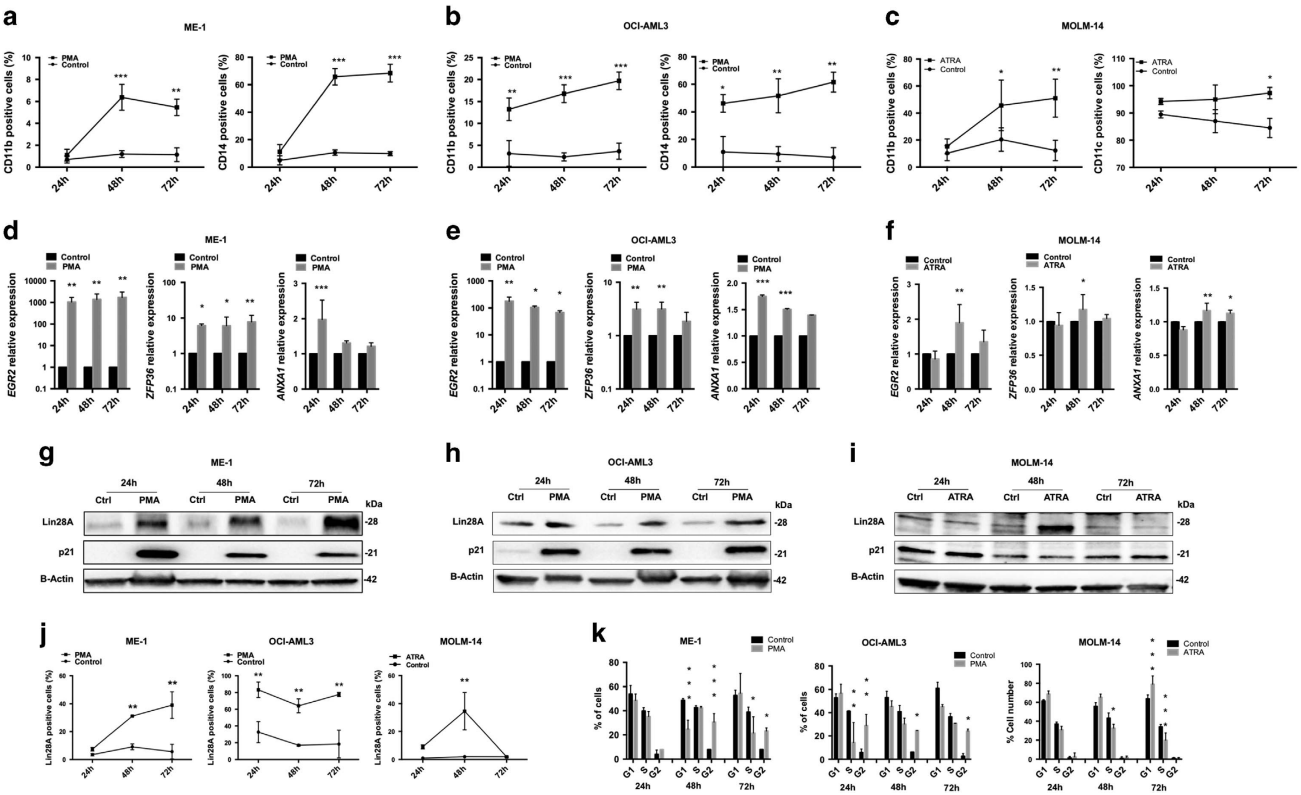

Figure 3 Lin28A upregulation during macrophage-like differentiation in AML cell lines. (**a** and **b**) Percentage of CD11b+ and CD14+ cells in ME-1 (**a**) and OCI-AML3 (**b**) after 24, 48 and 72 h of treatment with PMA, by cytofluorimetric analysis. (**c**) Percentage of CD11b+ and CD11c+ cells of MOLM-14 after 24, 48 and 72 h of treatment with ATRA, by cytofluorimetric analysis. (**d–f**) qRT-PCR of *EGR2, ZFP36* and *ANXA1* in ME-1 (**d**), OCI-AML3 (**e**) and MOLM-14 (**f**) after 24, 48 and 72 h of treatment with PMA or ATRA. (**g–i**) Western blotting (WB) analysis of Lin28A, p21 and β-actin in ME-1 (**g**), OCI-AML3 (**h**) and MOLM-14 (**i**) after 24, 48 and 72 h of treatment with PMA. (**j**) Percentage of Lin28A+ ME-1, OCI-AML3 and MOLM-14 cells after 24, 48 and 72 h of treatment with PMA or ATRA, by cytofluorimetric analysis. (**k**) Cell-cycle analysis in ME-1, OCI-AML3 and MOLM-14 cells after 24, 48 and 72 h of treatment with PMA or ATRA. The line and bar graphs represented mean $\pm$ S.D. from three independent experiments. Statistically significant analyses are indicated by asterisks: $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$

association with genotypic and phenotypic stratification. Moreover, we found a higher percentage of Lin28A+ cells in myeloid precursors compared with that in erythroid and lymphoid normal precursors, suggesting a preferential involvement of this protein in myeloid lineage differentiation.

Recently, Chaudhuri *et al.*[26] demonstrated that the knockdown of Lin28A in mouse hematopoietic system led to myeloid cell expansion and decrease of B-cell number, thus triggering an alteration of hematopoiesis. Furthermore, its overexpression in normal HSC produced a significant reduction of total white blood cells, causing mice dead at 5 weeks, probably because of the impaired hematopoietic development.[26]

Our data, instead, showed that Lin28A overexpression in AML cells activated myeloid maturation. We observed, in fact, an increase of myeloid differentiation markers and a cell-cycle arrest with p21 expression augment. Literature data demonstrated that p21, a cyclin-dependent kinase inhibitor, induced cell-cycle arrest if overexpressed in progenitor cells favoring macrophage differentiation because of the accumulation of PU.1, a lineage-determining factor.[40] Of importance, we also detected a significant increase of macrophage-specific genes like *early growth response 2* (*EGR2*), an EGR protein involved in macrophage growth and differentiation,[34,41] tristetraprolin (*ZFP36*), an anti-inflammatory and anticarcinogenic protein that is also involved in monocyte/macrophage differentiation processes and *annexin A1* (*ANXA1*) an anti-inflammatory protein stored in the macrophage cytosol.[37,42] In addition, we demonstrated that Lin28A is a positive regulator of granulocytic- and macrophage-like differentiation. In fact, we observed its significant increase simultaneously augmented different myeloid-specific markers, stimulated by ATRA or PMA treatment, in five AML cell lines with different genotype and morphology.

Previous studies reported that Lin28A is a direct target of *miR-128*,[28] a microRNA involved in hematopoiesis.[29,30] Different studies have associated *miR-128a* with leukemia, showing that *miR-128a* belongs to a set of miRNAs with stringent specificity for AML or ALL.[31–33] Moreover, *miR-128a* expression was found to be associated with a subgroup of AML patients with high-risk molecular features, refractoriness, relapse and death.[31,33]

In our study, we evaluated *miR-128a* expression in our cohort of AML patients. Of interest, *miR-128a* showed a significantly higher level in APL and AML with mature phenotypes harboring *FLT3* and/or other alterations. Qian *et al.*[28] sustained that *miR-128* directly target *BMI1, CSF1, KLF4, LIN28A, NANOG* and *SNAIL*. Some of these genes are involved in self-renewal (*Bmi1* and *Nanog*)[43] and

differentiation (*CSF1* and *KLF4*).[44,45] Similar to Lin28A, they are deregulated in AML.[45–47] KLF4, for example, a lineage-specific transcriptor factor that promotes monocyte differentiation is downregulated in undifferentiated subtype M0 and in FLT3-ITD and NPM1-mutant AML.[45] BMI1, instead, a polycomb group protein involved in self-renewal is overexpressed in different AML subtypes.[46] Given that gene regulation is complex and depend on different factors,[45,48–50] the relative

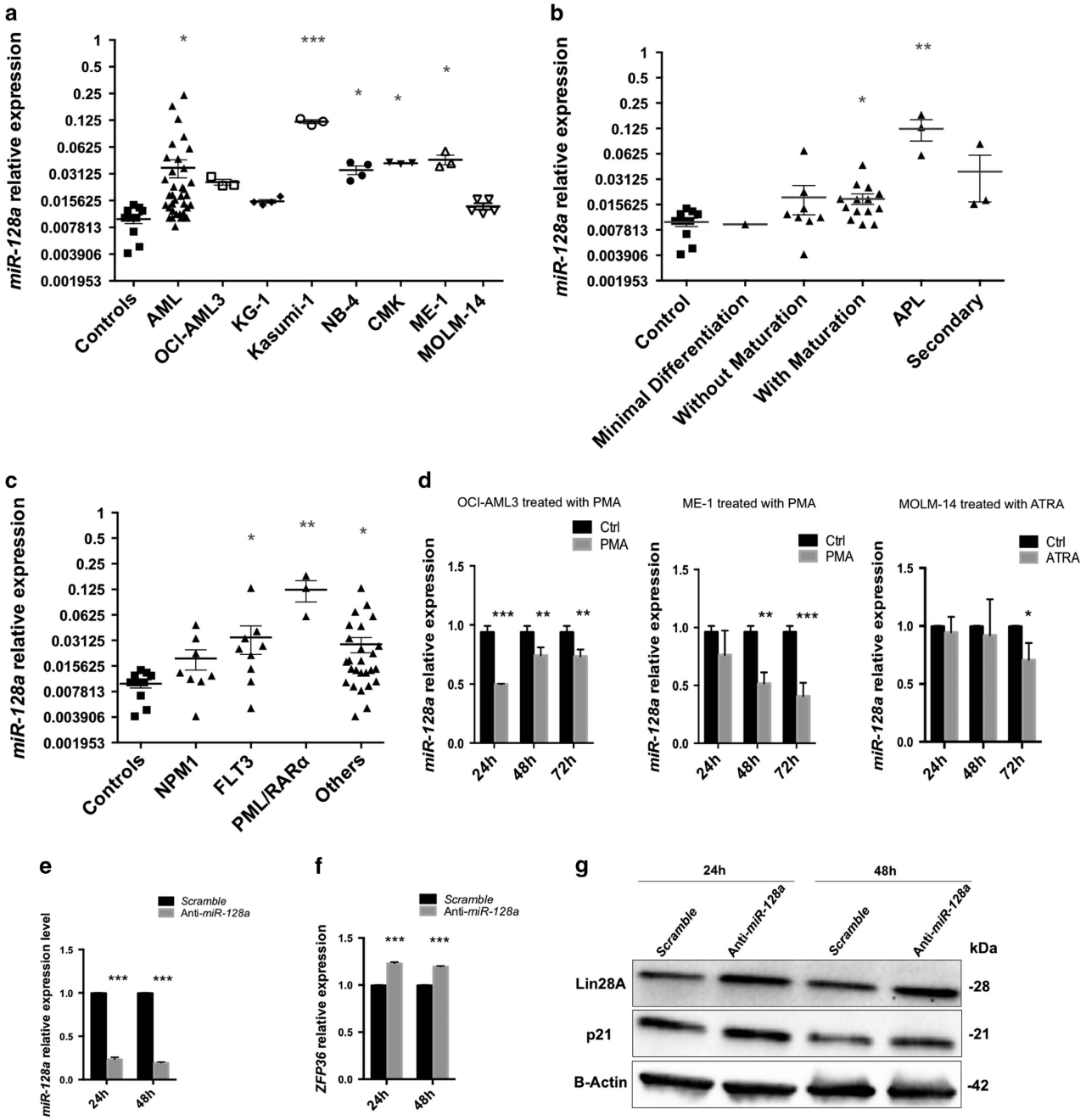

Figure 4 *MiR-128a* expression in leukemic blasts from AML patients and its inhibition in OCI-AML3 cell line. (**a**) qRT-PCR of *MiR-128a* in 10 healthy controls, 35 AML patients and 6 AML cell lines (OCI-AML3, KG-1, Kasumi-1, NB4, CMK, ME-1 and MOLM-14); *RNU44* was used for normalization. Relative values were calculated on the basis of the ΔCp method. Results are shown as mean ± S.E.M. (**b**) Expression of *miR-128a* in AML patients stratified for morphologic features (with minimal differentiation, $n=1$; without maturation, $n=8$; with maturation including: $n=3$ with maturation, $n=9$ acute myelomonocytic leukemia, $n=2$ acute monoblastic/monocytic leukemia; APL, $n=3$; secondary AML, $n=3$) was compared with 10 healthy controls. Results are shown as mean ± S.E.M. (**c**) Expression of *miR-128a* in AML patients with specific mutations (NPM1, $n=8$, FLT3, $n=9$ or with other alterations, $n=26$) was compared with 10 healthy controls. Results are shown as mean ± S.E.M. (**d**) qRT-PCR of *miR-128a* in OCI-AML3, ME-1 and MOLM-14 cells after 24, 48 and 72 h of treatment with PMA or ATRA. The bar graphs represented mean ± S.D. from three independent experiments. (**e** and **f**) qRT-PCR of *miR-128a* (**e**) and *ZFP36* (**f**) in OCI-AML3 after 24 and 48 h of scramble or anti-*miR-128a* transfection. The bar graphs represented mean ± S.D. from three independent experiments. (**g**) Western blotting (WB) analysis of Lin28A, p21 and β-actin in OCI-AML3 after 24 and 48 h of transfection with scramble or anti-*miR-128a*. Statistically significant analyses are indicated by asterisks: $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$

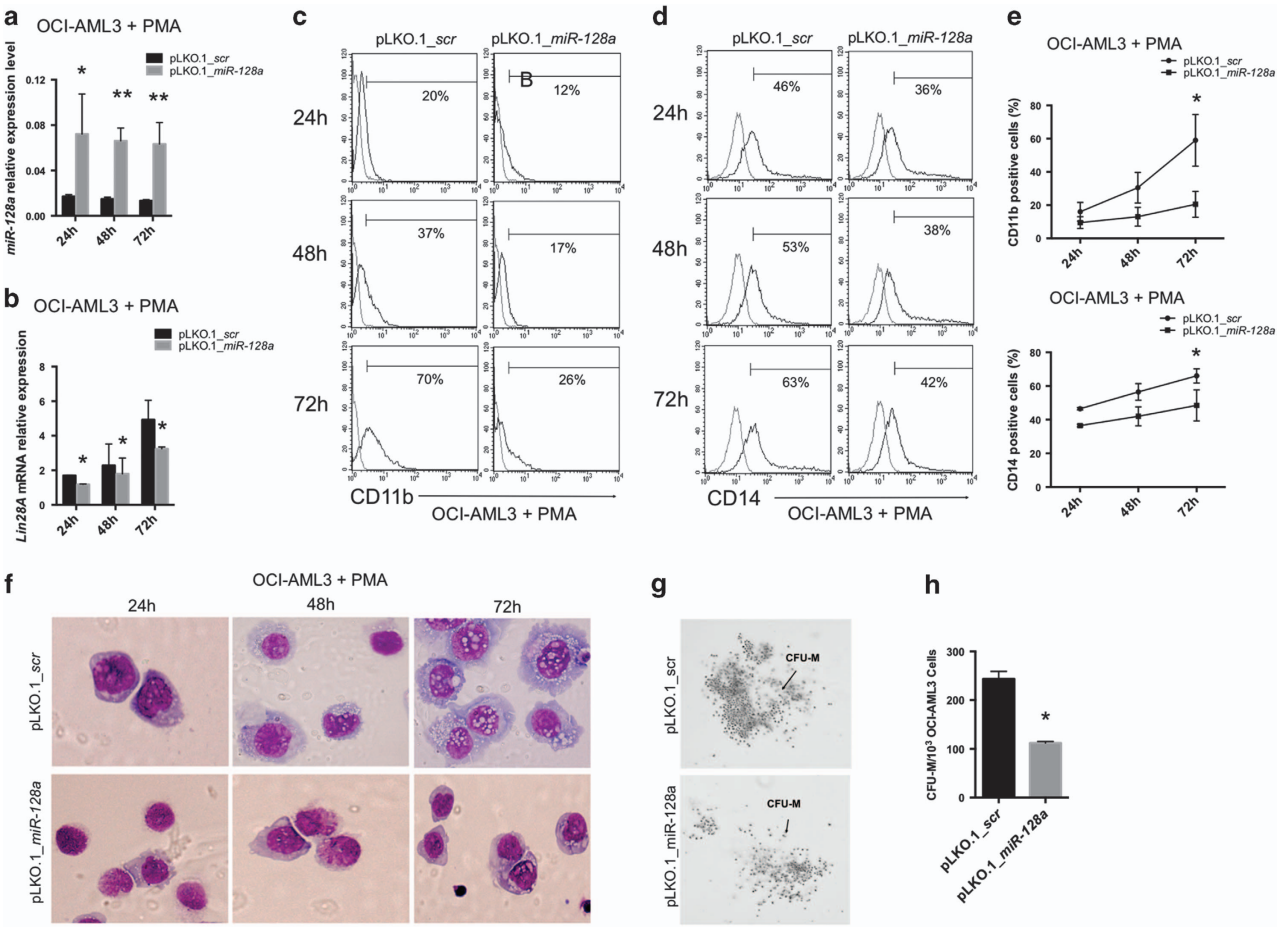

Figure 5 *MiR-128a* overexpression in OCI-AML3 cell line. (**a** and **b**) qRT-PCR of *miR-128a* (**a**) and *Lin28A* (**b**) in OCI-AML3 infected with pLKO.1_scr or pLKO.1_miR-128a after 24, 48 and 72 h of PMA treatment. (**c** and **d**) Representative histogram plots of CD11b+ (**c**) and CD14+ cells (**d**) in OCI-AML3 infected with pLKO.1_scr or pLKO.1_miR-128a after 24, 48 and 72 h of PMA treatment. (**e**) Percentage of CD11b+ and CD14+ OCI-AML3 cells infected with pLKO.1_scr or pLKO.1_miR-128a after 24, 48 and 72 h of PMA treatment, by cytofluorimetric analysis. (**f**) May–Grünwald Giemsa staining of OCI-AML3 infected with pLKO.1_scr or pLKO.1_miR-128a after 24, 48 and 72 h of PMA treatment. (**g**) Colony-forming assay of OCI-AML3 after infection with pLKO.1_scr or pLKO.1_miR-128a. Colonies were observed at day 14 of the semisolid culture under × 20 magnification. (**h**) Count of CFU-M colonies. The line and bar graphs represented mean ± S.D. from three independent experiments. Statistically significant analyses are indicated by asterisks: $^{*}P < 0.05$ and $^{**}P < 0.01$

upregulation of *miR-128* could not be sufficient to repress all these genes.

Various microRNAs have an important role in acute myeloid leukemogenesis,[50,51] because of their role in the different stages of hematopoiesis.[29,52] *MiR-125b*, for example, is overexpressed in certain types of AML (C/EBPα, t(2;11)(p21;q23), GATA1) and inhibits myeloid differentiation.[50,53] Moreover, its overexpression causes a dose-dependent myeloproliferative disorder progressing to a lethal myeloid leukemia in mice.[50] *MiR-181* family, instead, was found abnormally upregulated in AML patients, with t(8;21) and t(15;17) inhibiting granulocytic- and macrophage-like differentiations.[54] Here, we demonstrated that *miR-128a* was downregulated during induced granulocyte- and macrophage-like differentiation of AML cell lines. Moreover, we showed a reduction of Lin28A- and myeloid-specific marker expression following enforced *miR-128a* expression, in spite of PMA treatment *in vitro*. Conversely, *miR-128a* transient inhibition in two cell lines enhanced myeloid maturation and Lin28A overexpression. Given the higher expression of *miR-128a* in AML with mature phenotypes and with *FLT3* or *PML/RARα* alterations, we decided to inhibit *miR-128a* maturation in leukemic cells of these subsets of patients to stimulate further propensity to cell differentiation. In fact, Lenti-miRZip-128a infection remarkably repressed *miR-128a* and improved granulocytic/macrophage-like differentiation in BM-derived AML blasts. Finally, we detected an augment of *Lin28A* in all infected AML blasts patients, while an increase of macrophage-specific genes occurred only in AML with *FLT3* mutation and mature phenotypes.

Specific microRNAs with established oncogenic functions, such as *miR-155*, *miR-125b*, *miR-181* and *miR-128a*, appear to be associated with particular AML subtypes.[31,50,55] Selected sets of microRNAs could be used as a target therapy tailored to specific biological and molecular features of AML.[50] In particular, we hypothesize that in AML subtypes with t(8;21) and inv16, differentiation block could be released by *miR-128a* knockdown in combination with differentiation agents. In this setting, we previously demonstrated that G-CSF treatment of a

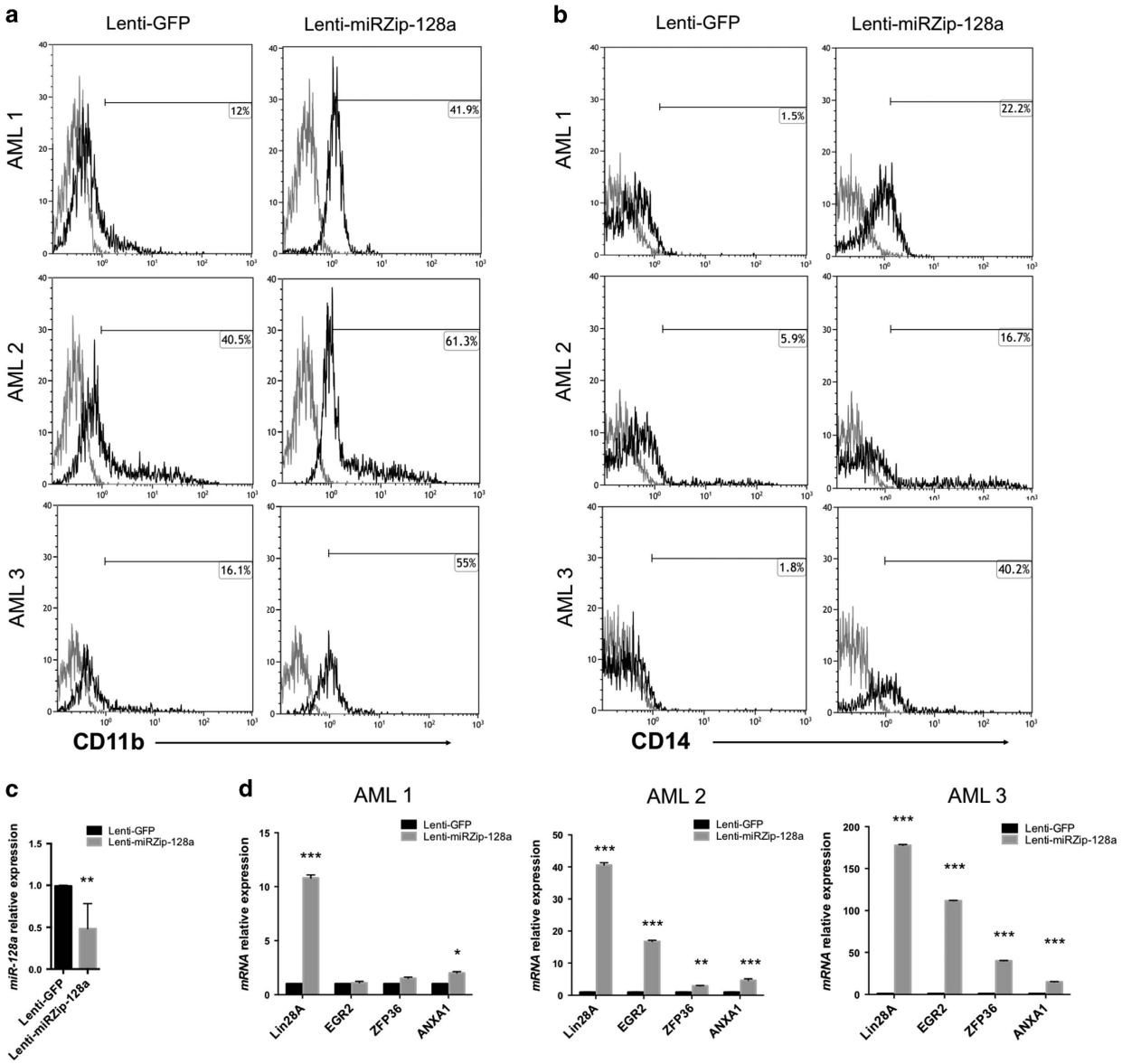

Figure 6 Inhibition of *miR-128a* in leukemic cells from AML patients. (**a** and **b**) Percentage of CD11b+ (**a**) and CD14+ cells (**b**) from three AML patients (AML1, AML2 and AML3) infected with Lenti-GFP or Lenti-miRZip-128a after 3 days of macrophage-like induction culture, by cytofluorimetric analysis. (**c**) qRT-PCR of *miR-128a* in AML patients infected with Lenti-GFP or Lenti-miRZip-128a. (**d**) qRT-PCR of *Lin28A*, *EGR2*, *ZFP36* and *ANXA1* in AML patients infected with Lenti-GFP or Lenti-miRZip-128a after 3 days of macrophage-like induction culture. Statistically significant analyses are indicated by asterisks: $*P<0.05$, $**P<0.01$ and $***P<0.001$

patient with t(8;21) AML led to complete remission.[56] Moreover, the combined inhibition of *miR-128a* and *miR-155* could be evaluated as a therapeutic option in high -isk AML patients harboring *FLT3* mutation.

In conclusion, we revealed a new regulatory axis *miR-128a/Lin28A* that affects hematopoiesis, favoring AML development. Our experiments suggest that the inhibition of *miR-128a* could provide a new strategy for AML therapy.

Materials and Methods

Human samples. BM samples were obtained from 40 AML patients (37 *de novo* and 3 secondary AML) at the time of diagnosis from the IRCCS CROB Hospital. The clinical and biological characteristics of AML patients are summarized in Supplementary Table S1. All patients gave written informed consent according to the Declaration of Helsinki. BM and peripheral blood samples of 13 healthy donors were also obtained from San Luigi Gonzaga Hospital of Turin. CD34+ cells of all samples were purified from mononuclear cells with a CD34 Microbead Kit (Miltenyi Biotec, Auburn, CA, USA). The purity of immunoselected cells routinely ranged between 90 and 95% and it was assessed by flow cytometric analysis using an allophycocyanin (APC) anti-CD34 (BD Pharmingen, San Jose, CA, USA).

Cell lines. The human AML cell lines, OCI-AML3, KG-1, Kasumi-1, NB4, CMK, ME-1 and MOLM-14, were acquired from American Type Culture Collection (Rockville, MD, USA) or Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). AML cell line characteristics were reported in Supplementary Table S2. OCI-AML3 cells were maintained in DMEM medium (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (FBS) (Gibco), 1% of penicillin–streptomycin (Gibco) and 4 mM of

L-glutamine (Gibco). KG-1, Kasumi-1, NB4, CMK, ME-1 and MOLM-14 cells were maintained in RPMI-1640 medium (Gibco) supplemented with 10% FBS, 1% of penicillin–streptomycin (Gibco) and 2 mM of L-glutamine (Gibco). Cells were grown at 37 °C in 5% CO_2.

Cell line differentiation assessment. Macrophage- or granulocytic-like differentiation was induced in OCI-AML3 and ME-1 cell lines with PMA (Sigma-Aldrich, St. Louis, MO, USA) at 100 nM concentration and in NB4 and KG-1 cells with ATRA (Sigma-Aldrich) at 10 μM concentration. Cells were seeded at 400 000/ml and were harvested after 24, 48 and 72 h to evaluate cell differentiation.

Induction culture of AML CD34+ cells. AML CD34+ cells were cultured in StemMACS HSC Expansion medium with StemMACS HSC Expansion Cocktail 1x (Miltenyi Biotec). To induce macrophage-like differentiation 20 ng/ml M-CSF and 1 ng/ml IL-6 (Miltenyi Biotec) were used.

Flow cytometry. Cytofluorimetric analysis of intracellular Lin28A protein levels was performed after fixation and permeabilization with the IntraCell Kit (Immunostep, Salamanca, Spain) followed by labeling with Lin28A (Cell Signaling Technology, Danvers, MA, USA) or its isotypic control (Cell Signaling Technology) in 11 BM healthy subjects and 9 AML patients. Lin28A protein expression was also evaluated in myeloid, lymphoid and erythroid precursors of CD34+ cells of healthy subjects by using the following fluorochrome-conjugated monoclonal antibodies and their specific isotypic controls: peridin chlorophyll (PerCP) anti-CD45, phycoerythrin (PE) anti-CD33, PE anti-CD19 and PE anti-CD71 (BD Pharmingen). The expression of myeloid-specific antigens CD14, CD11b and CD15 on cell surface was determined by direct immunofluorescent staining with the following fluorochrome-conjugated monoclonal antibodies and their specific isotypic controls: APC anti-CD14, PE anti-CD11b, PE anti-CD11c and PerCP anti-CD15 (BD Pharmingen). For cell-cycle analysis, cells were fixed and permeabilized, and then labeled with PI/RNase staining solution for 30 min. Cells were acquired by FACS Calibur (BD) and analysis was performed using the ModFit LT Software (Verity Software House, Topsham, ME, USA).

***In vitro* transfection of AML cell lines.** Lin28A transfections were performed in OCI-AML3 by using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's procedure. Transient transfection of *anti-miR-128a* molecule (300 pmol) and negative control (Ambion, Applied Biosistem, Foster City, CA, USA) was accomplished in OCI-AML3 and ME-1 cell lines with Lipofectamine RNAi Max (Life Technologies) in accordance with the manufacturer's procedure.

RNA isolation and qRT-PCR for mRNA and miRNA quantification. Mononuclear cells were obtained by Ficoll-Paque gradient centrifugation. Total RNA was extracted using Trizol reagent (Life Technologies) according to the manufacturer's instructions. Reverse transcription was performed using 1 μg of total RNA from each sample by High Capacity cDNA Reverse Transcription Kit (Applied Biosistem, Foster City, CA, USA). qRT-PCR was performed as described previously.[57] Simultaneous quantification of *ABL1* mRNA was used as a reference for mRNA TaqMan assay data normalization. *miR-128* expression was normalized on *RNU44*. The comparative cycle threshold (Ct) method for relative quantification of mRNA and miRNA expression (User Bulletin No. 2; Applied Biosystems) was used to determine transcript levels.

Western blotting. Cells were lysed as reported previously.[58] Total proteins were extracted from AML cell lines. Equal amount of protein extract (60 μg) was transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked for 1 h with 5% milk (Sigma-Aldrich) at room temperature, and then incubated with primary antibodies directed toward Lin28A (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p21 (Merck Millipore, Billerica, MA, USA) and β-actin (Sigma-Aldrich), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad). Protein bands were visualized and quantified as described previously.[59]

Lentivirus production and infection. *MiR-128a* expression vector were made by cloning ~ 60 bp 5′ and 3′ of the pre-miRNA into the multiple cloning site for pLKO.1 (Addgene, Cambridge, MA, USA). Lenti_GFP control and Lenti-miRZip-128a were purchased by System Biosciences (Palo Alto, CA, USA). The virus packaging was performed according to the manufacturer's instructions. The virus particles (lenti_128a, lenti_GFP control and Lenti-miRZip-128a) were harvested and concentrated using PEG-it Virus Precipitation Solution (System Biosciences). Virus titer was determined in 293TN cells using the global Ultrarapid Lentiviral Titer Kit (System Biosciences). For transduction, AML primary cells and OCI-AML3 were seeded onto 6-well plates at 800 000 cells per ml. Cells were infected with lentiviral stocks at an MOI of 5 in the presence of polybrene. AML primary cells were sorted for the expression of GFP using cell sorter MoFlo Atrios (Beckman Coulter, Brea, CA, USA). OCI-AML3 cells were maintained with puromycin 0.5 μg/ml.

Colony-forming assay. OCI-AML3 cells infected with pLKO.1_scr or pLKO.1_miR-128a were cultured in 35mm dishes in MethoCult Classic (Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer's instruction. CFU-M were visualized, measured and counted after being cultured in incubator at 37 °C for 14 days.

May–Grünwald Giemsa staining. OCI-AML3 cells infected with pLKO.1_scr or pLKO.1_miR-128a were harvested at 24, 48 and 72 h after PMA treatment and stained with May–Grünwald for 5 min and Giemsa for 30 min. The cell smears were washed with water, air-dried and observed under optical microscopy (Leica, Wetzlar, Germany).

Statistical analysis. Results are shown as mean $\pm$ S.D. or S.E.M. Mann–Whitney *U*-test was used to analyze two group comparisons (protein expression qRT-PCR). Analyses of multiple groups (qRT-PCR of *Lin28A* and *miR-128* in patients and cell lines, *Lin28A* data set analysis) were performed by Dunn's multiple comparisons test after one-way ANOVA with Kruskal–Wallis test. Cytofluorimetric analyses (time course) and qRT-PCR at different time points were carried out by two-way ANOVA followed by *post hoc* multiple comparisons using Sidak's test. For all tests, a *P*-value < 0.05 was taken as statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. This work was supported by Italian ministry of Health, Current Research Funds for IRCCS (CUP E66J12000230001).

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Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)