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

Luciana De Luca*1,9, Stefania Trino1,9, Ilaria Laurenzana1, Daniela Tagliaferri2, Geppino Falco2, Vitina Grieco3, Gabriella Bianchino3, Filomena Nozza3, Valentina Campia3, Francesca D’Alessio5, Francesco La Rocca1, Antonella Caivano5, Oreste Villani6, Daniela Cillonì4, Pellegrino Musto7,9 and Luigi Del Vecchio5,8,9

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 cells14,15 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-128,29 a microRNA able to hold hematopoietic cells in an early progenitor stage, blocking their

1Laboratory of Preclinical and Translational Research, IRCCS – Referral Cancer Center of Basilicata (CROB), Rionero in Vulture 85028, Italy; 2Biogem Scarl, Istituto di Ricerca Genetica ‘Gaetano Salvatore’, Ariano Irpino 83031, Italy; 3Laboratory of Clinical Research and Advanced Diagnostics, IRCCS – Referral Cancer Center of Basilicata (CROB), Rionero in Vulture 85028, Italy; 4Department of Clinical and Biological Sciences, University of Turin, Orbassano 10043, Italy; 5CEINGE Biotecnologie Avanzate scrl, Naples 80147, Italy; 6Department of Onco-Hematology, IRCCS – Referral Cancer Center of Basilicata (CROB), Rionero in Vulture 85028, Italy; 7Scientific Direction, IRCCS – Referral Cancer Center of Basilicata (CROB), Rionero in Vulture 85028, Italy and 8Department of Molecular Medicine and Medical Biotechnologies, University of Naples Federico II, Napoli 80138, Italy

*Corresponding author: L De Luca, Laboratory of Preclinical and Translational Research, IRCCS – Centro di Riferimento Oncologico della Basilicata (CROB), via Padre Pio 1, Rionero in Vulture 85028, Italy. Tel: +39 0972 726528; Fax: +39 0972 723509; E-mail: dr.luciana.deluca@gmail.com

9These authors contributed equally to this work.

Received 14.2.17; revised 05.5.17; accepted 05.5.17; Edited by M Diederich
differentiation towards more mature cells. Moreover, this microRNA was found associated with AML. 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 profiles, 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 myeloid/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). And ANXA1, a gene normally stored in inside macrophage cytosol (Figure 2l) 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). 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–j) 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 2i), 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 2i).

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/RARA and other genetic 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-AF9.39 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
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-128a/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 cytometric 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.
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.1,2,39 In this study, we showed that Lin28A, an RNA-binding protein,12 was significantly underexpressed in AML samples without any
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. 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.45 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. 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) and

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-i) 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 ± S.D. from three independent experiments. Statistically significant analyses are indicated by asterisks: *P<0.05, **P<0.01 and ***P<0.001
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 = 6; 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
upregulation of miR-128 could not be sufficient to repress all these genes.

Various microRNAs have an important role in acute myeloid leukemogenesis,\textsuperscript{50,51} because of their role in the different stages of hematopoiesis.\textsuperscript{29,52} MiR-125b, for example, is overexpressed in certain types of AML (C/EBP\textalpha, t(2;11)(p21;q23), GATA1) and inhibits myeloid differentiation.\textsuperscript{50,53} Moreover, its overexpression causes a dose-dependent myeloproliferative disorder progressing to a lethal myeloid leukemia in mice.\textsuperscript{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.\textsuperscript{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/RAR\textalpha 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, LentivirusZip-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.\textsuperscript{51,50,55} Selected sets of microRNAs could be used as a target therapy tailored to specific biological and molecular features of AML.\textsuperscript{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 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 cytometric analysis. (f) May–Grunwald 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 x 20 magnification. (h) Count of CFU-M colonies. The line and bar graphs represented mean \( \pm \) S.D. from three independent experiments. Statistically significant analyses are indicated by asterisks: *\( P<0.05 \) and **\( P<0.01 \)
A patient with t(8;21) AML led to complete remission. Moreover, the combined inhibition of miR-128a and miR-155 could be evaluated as a therapeutic option in high-risk 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...
performed in OCI-AML3 by using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) and penicillin–streptomycin (Gibco) and 2 mM of l-glutamine (Gibco). Cells were grown at 37 °C in 5% CO₂.

**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 Intracellular Kit (Immuno-step, 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: peridinin 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.

**Lentivirus production and infection.** MR-128a expression vector was made by cloning – 60 bp 5’ and 3’ of the pre-miRNA into the multiple cloning site for pLKO.1 (Addgene, Cambridge, MA, USA). Lent-GFP control and Lent-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 293T cells using the global Ultralow 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 Atios (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 instructions. 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 ± 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 IRCSS (CUP E66J1200230001).

1. Papaemmanuil E, Gerstung M, Bullinger L, Gaidzik VI, Paschka P, Roberts ND et al. Genomic classification and prognosis in acute myeloid leukemia. N Engl J Med 2016; 374: 2209–2221.
2. Dohner H, Weisdorf DJ, Bloomfield CD. Acute myeloid leukemia. N Engl J Med 2015; 373: 1136–1152.
3. De Kouchkovsky I, Abdul-Hay M. Acute myeloid leukemia: a comprehensive review and 2016 update. Blood Cancer J 2016; 6: e441.
4. Arber DA, Orazi A, Hasselerian RP, Theile J, Borowitz MJ, Le Beau MM et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood 2016; 127: 2391–2405.
5. Fukushima N, Minami Y, Kakuchi S, Kuwatsuma Y, Hayakawa F, Jamieson C et al. Small-molecule Hedgehog inhibitor attenuates the leukemia-initiation potential of acute myeloid leukemia cells. Cancer Sci 2016; 107: 1422–1429.
6. Lu FL, Yu CC, Chiu HH, Lu HE, Chen SY, Lin S et al. Sonic hedgehog antagonists induce cell death in acute myeloid leukemia cells with the presence of lipopolysaccharides, tumor necrosis factor-alpha, or interferons. Invest New Drugs 2013; 31: 823–832.
7. Chang E, Ganguly S, Rajkhowa T, Groke CD, Lewis M, Konig H. The combination of FLT3 and DNA methyltransferase inhibition is synergistically cytotoxic to FLT3/ITD acute myeloid leukemia cells. Leukemia 2016; 30: 1025–1032.
8. Taskesen E, Staal FJ, Reinders MJ. An integrated approach of gene expression and DNA-methylation profiles of WHN signaling genes uncovers novel prognostic markers in acute myeloid leukemia. BMC Bioinform 2015; 16(Suppl 4): S4.
9. Ye Q, Jiang J, Zhan G, Yan W, Huang L, Hu Y et al. Small molecule activation of NOTCH signaling inhibits acute myeloid leukemia. Scientific Rep 2016; 6: 26510.
10. Takam Kamga P, Bassi G, Cassaro A, Mitofo M, Di Trapani M, Gatti A et al. Notch signaling drives bone marrow stromal cell-mediated chemoresistance in acute myeloid leukemia. Oncotarget 2016; 7: 21713–21727.
11. Gu Y, Masiero M, Banham AH. Notch signaling: its roles and therapeutic potential in hematological malignancies. Oncotarget 2016; 7: 29804–29823.
12. Jiang SJ, Baltimore D. RNA-binding protein Lin28 in cancer and immunity. Cancer Lett 2016; 375: 108–113.
13. Zhou J, Ng SB, Chng WJ. LIN28/LIN28B: an emerging oncogenic driver in cancer stem cells. Int J Biochem Cell Biol 2013; 45: 973–978.
14. Shyh-Chang N, Daley GQ. Lin28: primordial regulator of growth and metabolism in stem cells. Cell Stem Cell 2013; 12: 395–406.

15. Zhong X, Li N, Liang S, Huang Q, Cooijs G, Zhang L. Identification of microRNAs regulating reprogramming factor LIN28 in embryonic stem cells and cancer cells. J Biol Chem 2010; 285: 41961–41971.

16. Wu J, Vodovnik MA, Smuga-Otto K, Antoniowicz-Bourget J, Francl JL, Tian S et al. Induced pluripotent stem cell lines derived from human somatic cells. Science 2007; 318: 1917–1920.

17. Gilsanz T, Bachok JL, Yong J, Dreyfuss G. RNA-binding proteins and post-transcriptional gene regulation. FEBS Lett 2008; 582: 1177–1186.

18. Wibert M, Huguette SC, Kapeli K, Stark TJ, Liang TY, Chen SX et al. LIN28 binds messenger RNAs at GGA3A motifs and regulates splicing factor abundance. Mol Cell 2012; 48: 195–206.

19. Hafner M, Max KE, Bandaru P, Morozov P, Gerstberger S, Brown M et al. Identification of microRNAs bound and regulated by human LIN28 proteins and molecular requirements for RNA binding. RNA 2012; 18: 575–582.

20. Lukong KE, Chang KW, Khandjian EW, Richard S. RNA-binding proteins in human genetic disease. Trends Genet 2008; 24: 416–425.

21. Triboulet R, Proux M, Gregory RI. A single Let-7 microRNA bypasses LIN28-mediated repression. Cell Rep 2015; 13: 263–266.

22. Lichtenstein B, BMP Rep 2016; 49: 3–5. Finn MA, Butler TW et al. MiR-181 mediates cell differentiation by interrupting the Lin28 and let-7 feedback circuit. Cell Death Differ 2012; 19: 378–386.

23. Jiang X, Huang H, Li Z, Li Y, Wang X, Gurbuxani S. Identification of microRNAs regulating PU.1 and the cell cycle controls myeloid differentiation. Blood 2012; 120: 4325–4340.

24. Chouardhi A, So AM, Mehta A, Minisandam A, Sinha N, Jonsson VD et al. Oncogenic miR-125B regulates hematopoiesis by targeting the gene Lin28. Proc Natl Acad Sci USA 2012; 109: 4233–4238.

25. Yuan J, Nguyen CK, Liu X, Kanellopoulou C, Muljo SA. Lin28b represses adult bone marrow hematopoietic progenitors to mediate fetal-like lymphopoiesis. Science 2012; 335: 1105–1108.

26. Qian P, Banerjee A, Wu ZS, Zhang X, Wang H, Pandey V et al. Loss of SNAIL regulates miR-128-2 on chromosome 3p22.3 targets multiple stem cell factors to promote transformation of murine erythroid cells. Cancer Res 2012; 72: 6036–6050.

27. Georgantas RW III, Hildreth R, Morisot S, Alder J, Liu CG, Heimfeld S et al. MiR-128-2 on chromosome 3p22.3 targets multiple stem cell factors to promote miR-125b in acute myeloid leukemia is dependent on the miR-125B regulatory circuit. Blood 2014; 123: 3226–3239.

28. De Luca L, Trino S, Laurenza S, Iacovino V, Simeon M, Dinarello S et al. MicroRNA-155 in serum-derived extracellular vesicles as a potential biomarker for hematologic malignancies – a short report. Cell Oncol 2016; 40: 97–103.

29. Ferrara F, Di Noto R, Viola A, Russo C, Boccuni P, Costantini S et al. Complete remission in acute myeloid leukemia with 18q21 following treatment with G-CSF: flow cytometric analysis of in vivo and in vitro effects on cell maturation. Br J Haematol 1998; 106: 520–523.

30. De Luca L, Trino S, Laurenza S, Simeon V, Calico G, Raimondi S et al. MiRNAs and piRNAs from bone marrow mesenchymal stem cell extracellular vesicles induce cell survival and inhibit cell differentiation of cord blood hematopoietic stem cells: a new insight in transplantation. Oncotarget 2016; 7: 6766–6782.

31. De Luca L, Trino S, Laurenza S, Simeon V, Calico G, Raimondi S et al. MiRNAs and piRNAs from bone marrow mesenchymal stem cell extracellular vesicles induce cell survival and inhibit cell differentiation of cord blood hematopoietic stem cells: a new insight in transplantation. Oncotarget 2016; 7: 6766–6782.

32. De Luca L, Trino S, Laurenza S, Simeon V, Calico G, Raimondi S et al. MiRNAs and piRNAs from bone marrow mesenchymal stem cell extracellular vesicles induce cell survival and inhibit cell differentiation of cord blood hematopoietic stem cells: a new insight in transplantation. Oncotarget 2016; 7: 6766–6782.

33. De Luca L, Trino S, Laurenza S, Simeon V, Calico G, Raimondi S et al. MiRNAs and piRNAs from bone marrow mesenchymal stem cell extracellular vesicles induce cell survival and inhibit cell differentiation of cord blood hematopoietic stem cells: a new insight in transplantation. Oncotarget 2016; 7: 6766–6782.

34. De Luca L, Trino S, Laurenza S, Simeon V, Calico G, Raimondi S et al. MiRNAs and piRNAs from bone marrow mesenchymal stem cell extracellular vesicles induce cell survival and inhibit cell differentiation of cord blood hematopoietic stem cells: a new insight in transplantation. Oncotarget 2016; 7: 6766–6782.

35. De Luca L, Trino S, Laurenza S, Simeon V, Calico G, Raimondi S et al. MiRNAs and piRNAs from bone marrow mesenchymal stem cell extracellular vesicles induce cell survival and inhibit cell differentiation of cord blood hematopoietic stem cells: a new insight in transplantation. Oncotarget 2016; 7: 6766–6782.

36. De Luca L, Trino S, Laurenza S, Simeon V, Calico G, Raimondi S et al. MiRNAs and piRNAs from bone marrow mesenchymal stem cell extracellular vesicles induce cell survival and inhibit cell differentiation of cord blood hematopoietic stem cells: a new insight in transplantation. Oncotarget 2016; 7: 6766–6782.