

miR-101 suppresses the development of MLL-rearranged acute myeloid leukemia

Dysregulation of miRNA (a class of short non-coding RNA) has been observed in solid tumors and leukemia,^{1,2} and the role of miRNA in cancer development is largely context-dependent.² While several miRNA (e.g. miR-29b)³ have been functionally linked to acute myeloid leukemia (AML), their antitumor effects *in vivo* are not evident, possibly due to the complexity and diversity of miRNA-mediated gene regulation. It remains unclear how miRNA contribute to an aggressive phenotype in heterogenous AML. Using an integrated miRNA and mRNA expression analysis, here we uncover a miRNA-regulatory network composed of eight miRNA (i.e. miR-29a/b, miR-101, miR-222, miR-26b, miR-27b, miR-140 and miR-155) whose downregulation is associated with leukemia aggressiveness. We also define a tumor suppressive role for miR-101 in the development of *mixed-lineage leukemia (MLL)*-rearranged AML. Restoration of miR-101 expression significantly impedes leukemia initiation and progression through induction of cell cycle inhibitors and inhibition of genes associated with self-renewal and pro-survival in leukemic stem cells (LSC).

Oncogenic rearrangement of the *MLL* gene in hematopoietic stem/progenitor cells (HSPC) induces aberrant gene expression and confers a poor clinical prognosis in acute leukemias. Transformation by MLL fusion proteins is primarily mediated through direct upregulation of the *HOXA*-cluster genes and HOX co-factors such as MEIS1.⁴ HOXA9 and MEIS1 are the most essential downstream effectors of MLL fusion proteins, and we have previously reported that their co-expression is sufficient to predispose HSPC to malignant transformation and induce AML.⁵ Aberrant overexpression of HOXA9 and MEIS1 is required for the induction and maintenance of *MLL*-rearranged AML, where LSC have been functionally defined.^{4,5}

We and others have shown that MLL fusion proteins (e.g., MLL-AF9) can serve as an initiating event for oncogenic conversion of normal HSPC into pre-LSC, which subsequently acquire additional events upon transplantation into mice for the development of LSC.^{4,7} The self-renewal rate in a pre-leukemic clone determines subsequent tumorigenic potential *in vivo*. We have previously demonstrated that pre-LSC transformed from normal HSPC by MLL-AF9 have higher self-renewal potential and produce a more aggressive leukemia in mice with shorter latency than pre-LSC transformed by

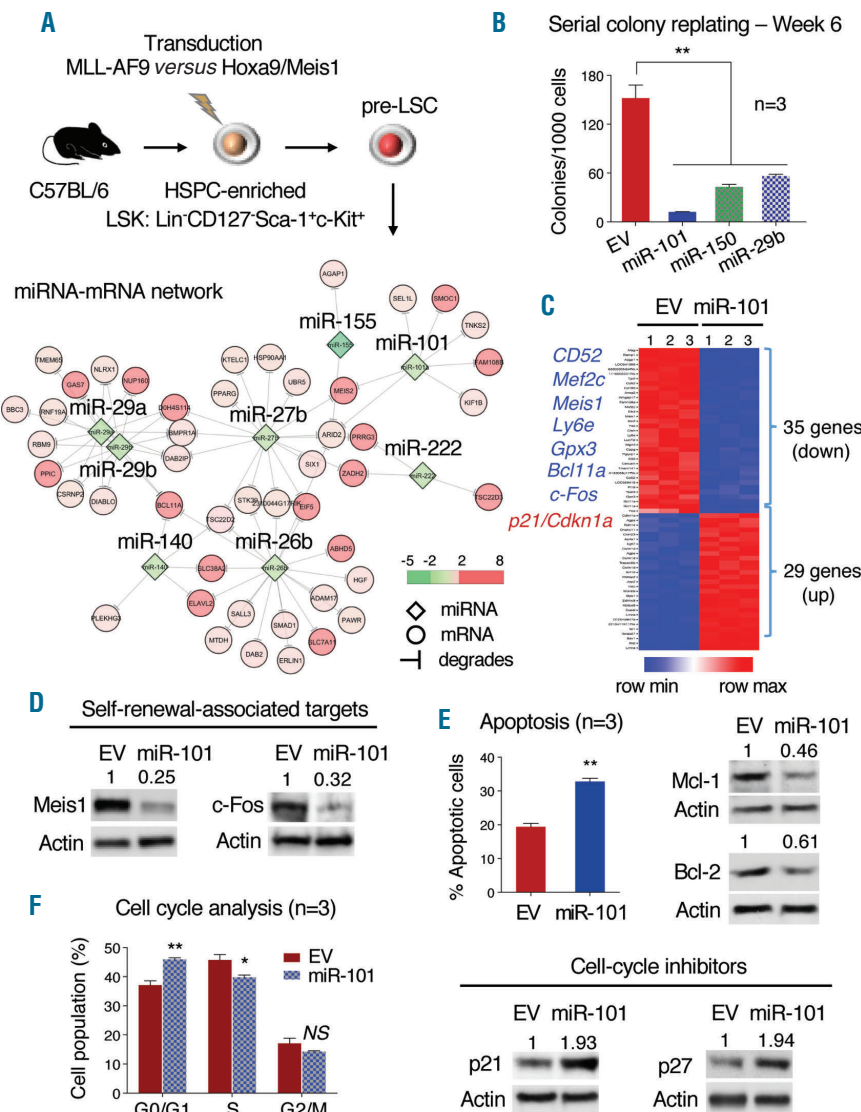


Figure 1. Overexpression of miR-101 impairs the function of MLL-AF9 pre-leukemic stem cells (pre-LSC). (A) Integrated genomic analysis identifying a tumor suppressor miRNA-regulatory network in MLL-AF9 pre-LSC compared to HOXA9/MEIS1 pre-LSC. Diamond shape: miRNA identified by Exiqon miRCURY LNA microRNA array. Circle: mRNA (targeted by miRNA) identified by Illumina expression array. (B) Serial colony replating assay of miRNA-expressing MLL-AF9 pre-LSC. The number of colonies per dish at the 6th round of replating is shown (n=3). (C) Heat map of microarray analysis showing differential gene expression in MLL-AF9 pre-LSC overexpressing miR-101 versus empty vector (EV) control (n=3) with a cut-off of the false discovery rate (FDR) ≤0.05 and fold change (FC) ≥1.5. (D) Western blot analysis confirming miR-101-induced downregulation of Meis1 and c-Fos expression in MLL-AF9 pre-LSC. (E) Percentage of apoptotic cells (n=3) in MLL-AF9 pre-LSC and western blot analysis showing reduced expression of Mcl-1 and Bcl-2 by miR-101 overexpression. (F) Cell cycle analysis of miR-101-expressing MLL-AF9 pre-LSC using Ki-67 and 7AAD staining (n=3 independent experiments) and western blot analysis revealing miR-10-induced upregulation of p21 and p27 expression. *P<0.05; **P<0.01; NS: not significant (P>0.05).

HOXA9/MEIS1.^{5,7} Despite major differences in self-renewal and mouse survival, pre-LSC mediated by MLL-AF9 and HOXA9/MEIS1 display similar immunophenotype and induce AML with similar histopathologic manifestations.^{5,7} Thus, genes differentially expressed in MLL-AF9 *versus* HOXA9/MEIS1 pre-LSC may contribute to the highly aggressive phenotype in MLL-AF9-induced AML.

To identify miRNAs that regulate the differential gene expression, we performed an integrated analysis for miRNA and mRNA expression profiling using the Bayesian Network with Splitting-Averaging strategy,⁸ and identified a tumor suppressive miRNA-regulatory network in HSPC-derived MLL-AF9 *versus* HOXA9/MEIS1 pre-LSC^{4,7} (Figure 1A and *Online Supplementary Figure S1A-D*). Of the eight miRNA identified, miR-101, miR-29b, miR-222 and miR-155 are

reportedly down-regulated in newly-diagnosed AML patient samples compared to normal human CD34⁺ HSPC, while miR-29a and miR-29b are down-regulated in patients with MLL-rearranged AML compared to other AML subtypes displaying a subtype-specific feature.⁹ These observations are consistent with our result showing lower levels of these miRNA in LSC (L-GMP, Lin⁻CD127⁻c-Kit⁺Sca1⁻GFP⁺CD16/32^{high}CD34⁺)⁴ flow-sorted from mice with MLL-AF9-induced AML than in normal murine HSPC (*Online Supplementary Figure S1E*), supporting a potential tumor suppressor role for the miRNA-regulatory network in MLL-rearranged AML.

Among the eight miRNA, miR-29b has been functionally defined as a tumor suppressor and its overexpression reduced tumorigenicity in a BCR-ABL-expressed K562 xenograft mouse model of human chronic myeloid

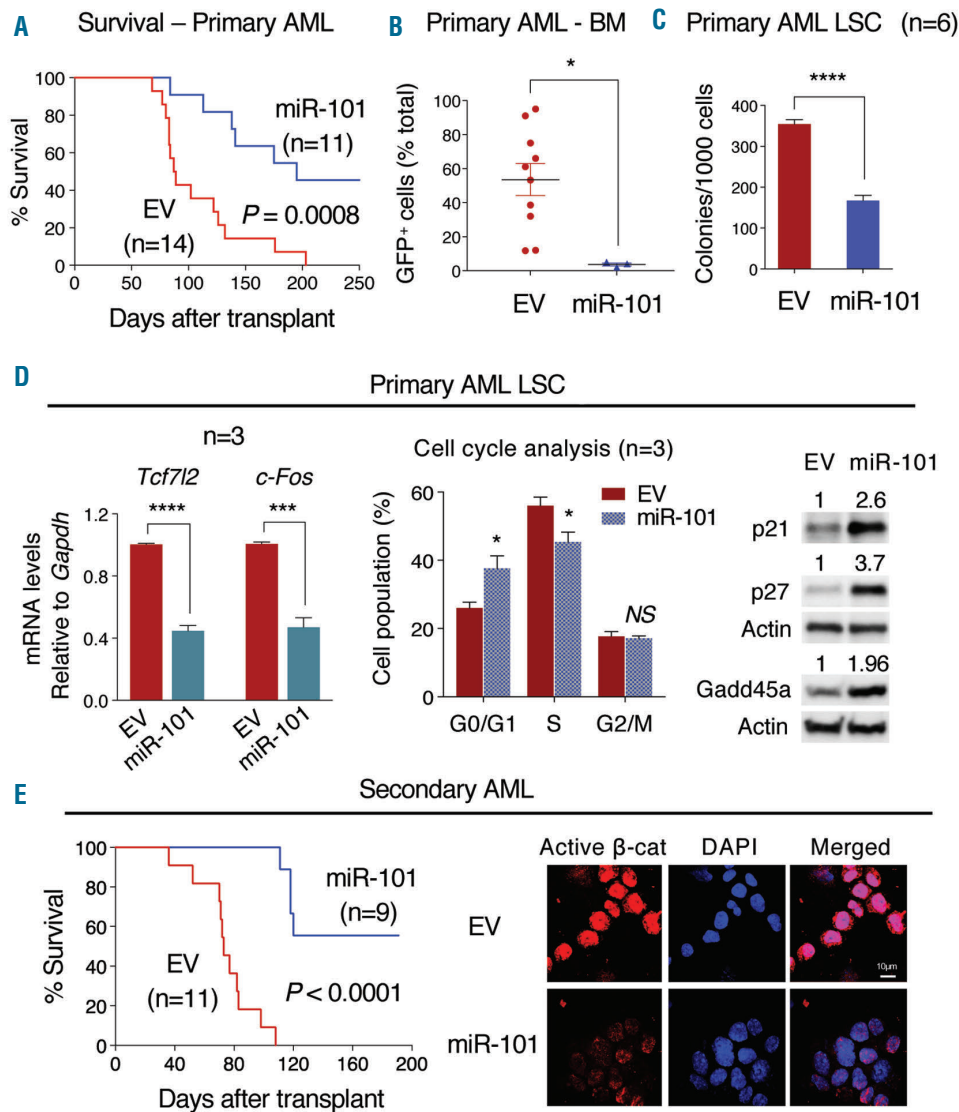


Figure 2. miR-101 suppresses the development of leukemic stem cells (LSC) in a mouse model of MLL-AF9-driven acute myeloid leukemia (AML). (A) Kaplan-Meier survival curves of mice receiving miR-101-expressing MLL-AF9 pre-LSC. 1×10^6 pre-LSC were transplanted into sublethally irradiated (6 Gy) BL6 recipient mice for the development of primary AML. *P*-values were determined by the log-rank test. (B) Percentages of GFP-positive (*) leukemic cells in the bone marrow (BM) of mice with primary AML. (C) Colony forming assay of MLL-AF9 LSC from primary AML. (D) Real-time quantitative polymerase chain reaction analysis of *Tcf7l2* and *c-Fos* gene expression, cell cycle analysis and expression of p21, p27, and Gadd45a proteins in primary MLL-AF9 LSC. (E) Kaplan-Meier survival curves of mice receiving GFP⁺ MLL-AF9 leukemic cells isolated from primary AML and confocal immunofluorescence showing miR-101-induced reduction of nuclear active β -catenin in leukemic cells from secondary AML. **P*<0.05; ****P*<0.0005; *****P*<0.0001; NS: not significant (*P*>0.05).

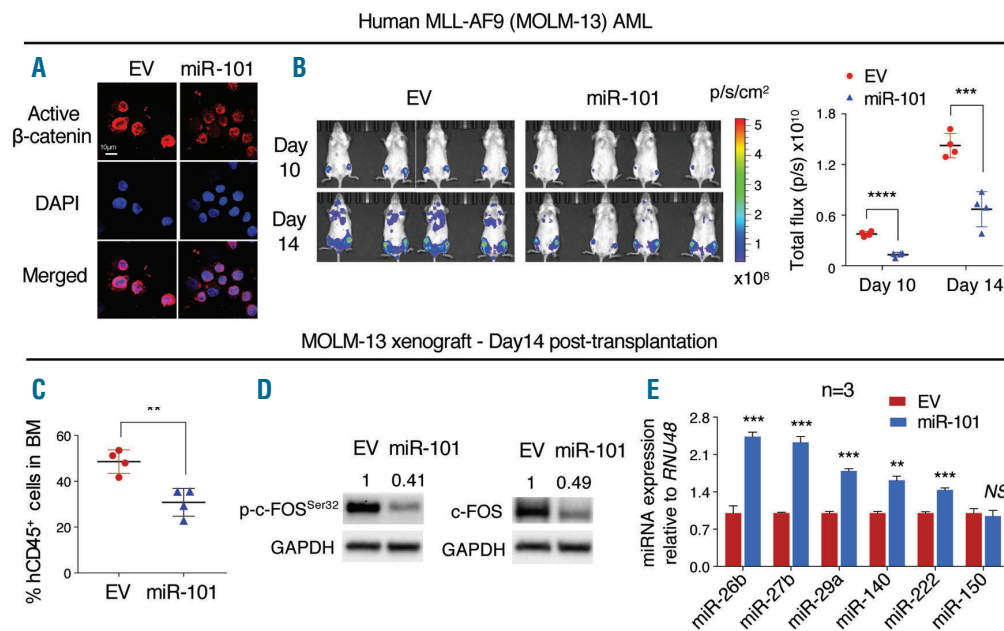


Figure 3. miR-101 reduces tumor burden in human MLL-AF9 (MOLM-13) xenografts. (A) Confocal immunofluorescence confirming miR-101-induced decrease of nuclear active β -catenin in human MLL-AF9 (MOLM-13) acute myeloid leukemia (AML) cells. (B) Bioluminescence imaging and total flux (photons/sec; p/s) of MOLM-13 xenograft mice ($n=4$). (C) Percentage of hCD45⁺ cells engrafted in the bone marrow (BM) of MOLM-13 xenograft mice. (D) Western blot analysis showing miR-101-induced reduction in p-c-FOS^{Ser32} and c-FOS levels. (E) Real time-quantitative polymerase chain reaction analysis of miRNA expression in miR-101-expressing human MLL-AF9 AML cells isolated from the BM of MOLM-13 xenograft mice. ** $P<0.01$; *** $P<0.0005$; **** $P<0.0001$; NS: not significant ($P>0.05$).

leukemia (CML) by targeting apoptosis, cell cycle and proliferation pathways.⁵ While miR-101 is reported as a putative tumor suppressor in several types of cancer *via* targeting diverse oncogenic pathways,¹⁰ its role in AML has not yet been explored. To further evaluate the miRNA-regulatory network, we investigated the tumor suppressive function of miR-101, which was one of the most down-regulated miRNA identified, in MLL-AF9-induced AML. We used miR-150 as a control because, despite not being in the network, its expression is down-regulated in both AML and CML.¹¹

HSPC-derived MLL-AF9 pre-LSC were transduced with a retroviral vector expressing miR-101, miR-29b or miR-150. Ectopic expression of these miRNA reduced the ability of pre-LSC to form colonies in serial replating assays (Figure 1B and *Online Supplementary Figure S2A and B*). Notably, miR-101 induced a significantly stronger growth-inhibitory effect on MLL-AF9 pre-LSC than miR-29b and miR-150, which was correlated with miR-101-mediated upregulation of cell-cycle inhibitor *p21/Cdkn1a* and downregulation of key Wnt/self-renewal target genes, including *Meis1*, *c-Fos*, *Mef2c*, *Bcl11a*, *CD52*, *Gpx3* and *Ly6e* identified by microarray analysis (Figure 1C). Subsequent RT-PCR and western blot analyses confirmed increased expression of p21 and reduced levels of Meis1, Bcl11a, c-Fos and Tcf712, which are known self-renewal genes in MLL-AF9-induced AML^{4,5,7} (Figure 1D and *Online Supplementary Figure S2C*). We and others have previously demonstrated that Wnt/ β -catenin signaling is required for the development of LSC in AML.⁵ Tcf712 and c-Fos are two key β -catenin transcriptional cofactors driving transcription of Wnt/ β -catenin target genes likely contributing to LSC self-renewal.⁷ Furthermore, the phenotypic defect in pre-LSC was accompanied by decreased cell

proliferation in methylcellulose and induced apoptotic cell death through suppression of pro-survival proteins Mcl-1 and Bcl-2, as well as G1 cell cycle arrest through elevation of cell-cycle inhibitors p21 and p27 (Figure 1E and F and *Online Supplementary Figure S2D*). These findings suggest that restoring expression of miR-101 impairs pre-LSC functions through regulation of genes associated with Wnt/self-renewal, pro-survival and cell cycle pathways.

We next assessed the inhibitory effect of miR-101 overexpression on AML development by intravenously transplanting miR-101-expressing MLL-AF9 pre-LSC into sublethally irradiated syngeneic recipient mice. Our data showed that enforced expression of miR-101 reduced the incidence and delayed the onset and progression of AML in mice, which was accompanied by a significant decrease in leukemic cell infiltration in bone marrow (BM) (Figure 2A and B). miR-101-expressing MLL-AF9 LSC flow-sorted from primary AML revealed reduced colony-forming capacity (Figure 2C and *Online Supplementary Figure S3*). This was likely caused by miR-101-induced suppression of Wnt target genes (*Tcf712* and *c-Fos*), and cell cycle arrest at the G1 phase *via* upregulation of p21, p27 and Gadd45a (Figure 2D). As a consequence of compromised LSC, miR-101-expressing AML cells from primary recipients generated a less aggressive leukemia in secondary recipient mice, whose BM cells exhibited a marked decrease in nuclear active β -catenin (Figure 2E). Altogether, these data underscore a tumor suppressor role for miR-101, whose ectopic expression impairs LSC development and reduces AML aggressiveness in mice.

We then investigated the effect of miR-101 overexpression on tumor burden in a mouse xenograft model of

human MLL-AF9 (MOLM-13) AML, which has an extremely short latency (14 days) and is an effective xenotransplantation model for *in vivo* functional studies. Consistent with our observation in murine MLL-AF9 leukemic cells, stable overexpression of miR-101 substantially decreased nuclear active β -catenin in MOLM-13 cells (Figure 3A and *Online Supplementary Figure S4A*). *In vivo* bioluminescence imaging showed that miR-101 overexpression reduced engraftment of human MOLM-13 leukemic cells in NOD/SCID/IL2R gamma-null (NSG) mice without affecting bone marrow homing (Figure 3B and C and *Online Supplementary Figure S4B*).

In agreement with miR-101-induced inhibition of leukemia cell proliferation *in vivo*, miR-101 markedly decreased the expression and phosphorylation of c-FOS (Figure 3D), a known β -catenin transcriptional co-factor implicated in the regulation of cell growth, survival, apoptosis, transformation and oncogenesis.¹² Phosphorylation of c-FOS is essential for its protein stabilization and maximal transactivation contributing to its increased cell-transforming activity.¹³ c-FOS is reportedly a direct target of several miRNA, including miR-101, miR-29 and miR-222.¹⁴ Our data confirmed a miR-101-mediated reduction in luciferase activity from the construct containing the c-FOS 3'-UTR (*Online Supplementary Figure S4C*). Enforced expression of exogenous c-FOS prevented miR-101-induced inhibition of leukemic cell viability (*Online Supplementary Figure S4D and E*). This result supports the role for c-FOS as a functional downstream target of miR-101.

Notably, restoration of miR-101 up-regulated the miRNA co-expression network, including miR-26b, miR-27b, miR-29a, miR-140 and miR-222, but did not alter the expression of miR-150 (Figure 3E). miR-101 is reportedly a direct regulator of histone methyltransferase EZH2 and likely regulates the miRNA network through modulation of EZH2-mediated H3K27me3. EZH2 augments MLL-AF9-initiated leukemogenesis by enhancing a myeloid differentiation block in AML.¹⁵ Our data showed that overexpression of miR-101 reduced levels of EZH2 and H3K27me3 in murine MLL-AF9 pre-LSC and human MLL-AF9 (MOLM-13) AML cells (*Online Supplementary Figure S5A*). Selective inhibition of EZH2 by EZH2 inhibitor EPZ-6438 caused a marked increase in miR-26b, miR-27b, miR-29b and miR-140 without affecting miR-150 (*Online Supplementary Figure S5B*), underlining epigenetic regulation of the miRNA network. Interestingly, we also observed EZH2 inhibitor-induced elevation of miR-101 (*Online Supplementary Figure S5B*). It is likely that there is a mutual regulation between miR-101 and EZH2-mediated H3K27me3. Increased miR-101 suppresses H3K27me3 by directly targeting EZH2; conversely, reduced H3K27me3 restores expression of the miRNA network including miR-101. These findings collectively suggest that miR-101 exerts its gene regulatory function in leukemogenesis, at least in part, by activating and co-operating with the network components. This co-operation between the miRNA allows for the simultaneous targeting of multiple oncogenic pathways that maximizes the tumor suppressive effect of miR-101.

In conclusion, we report here a miRNA-regulatory network associated with disease aggressiveness and demonstrate a tumor suppressor role for miR-101 in MLL-rearranged AML. Enforced expression of miR-101 impairs LSC self-renewal and restrains leukemia development in mice. Together with prior findings showing a tumor suppressor role for miR-29b in leukemia,⁵ our data support

the value of the miRNA co-expression network as potential novel targets for miRNA-based therapies in AML.

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