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

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Supplementary information

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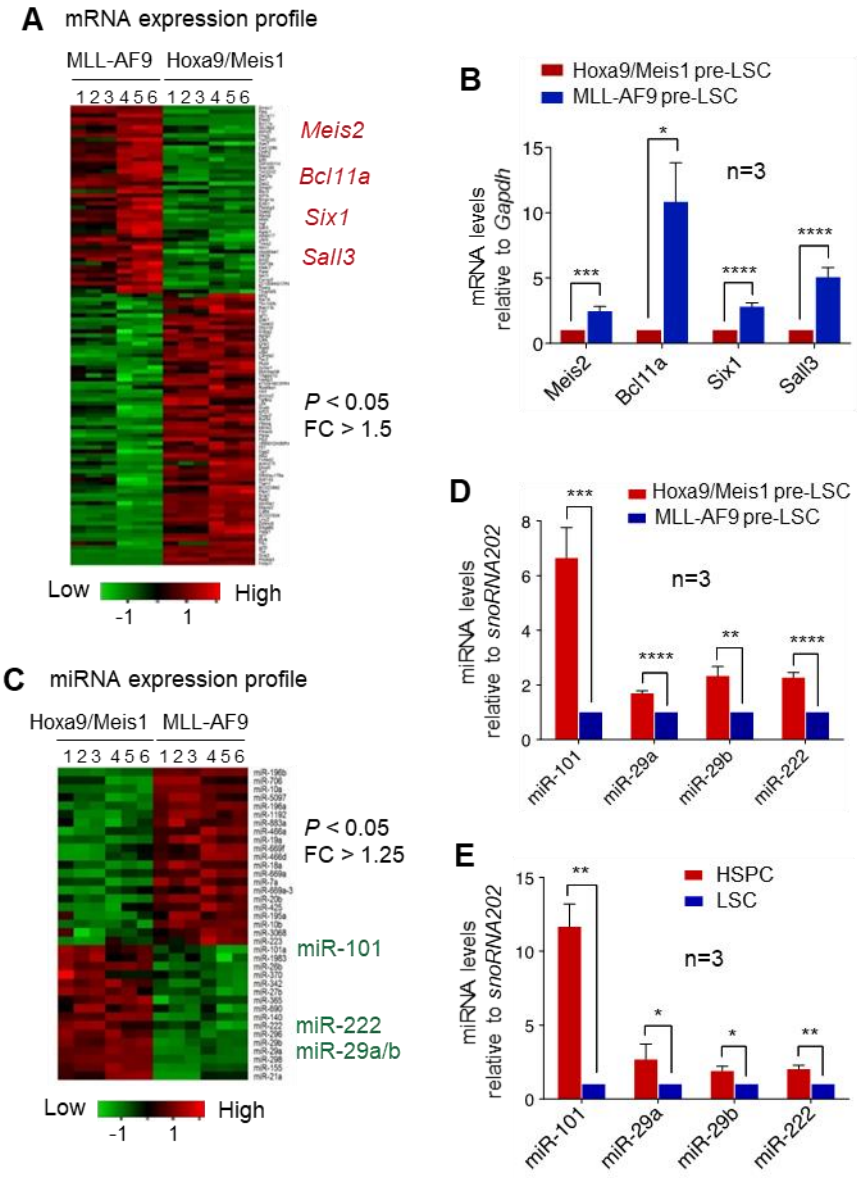
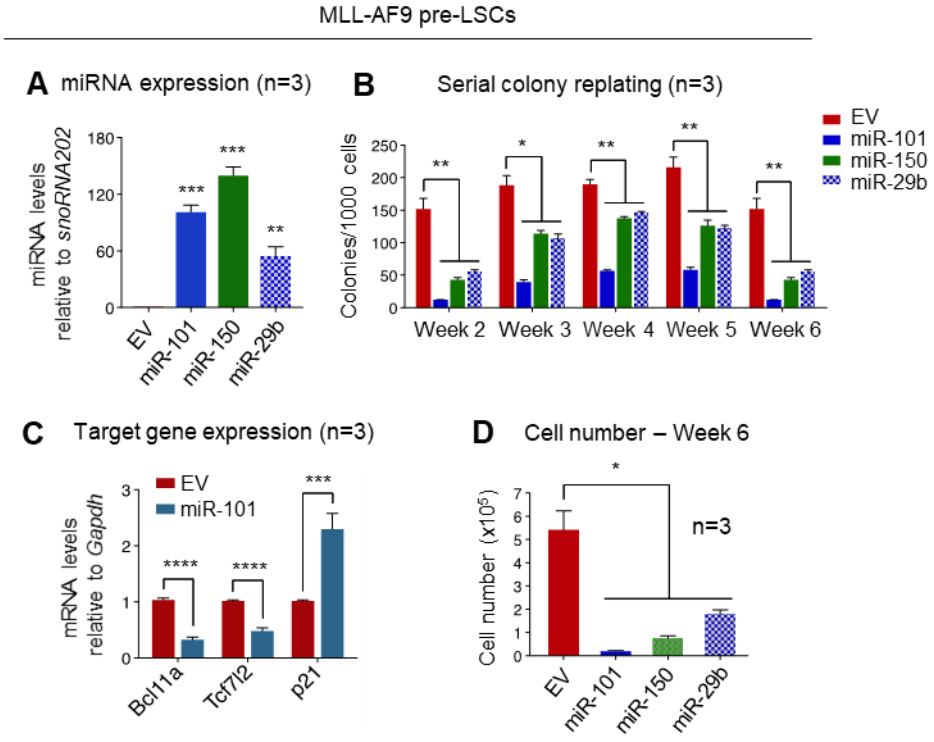
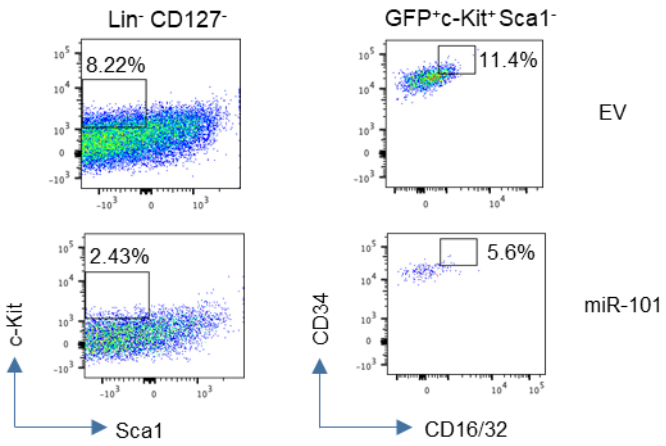


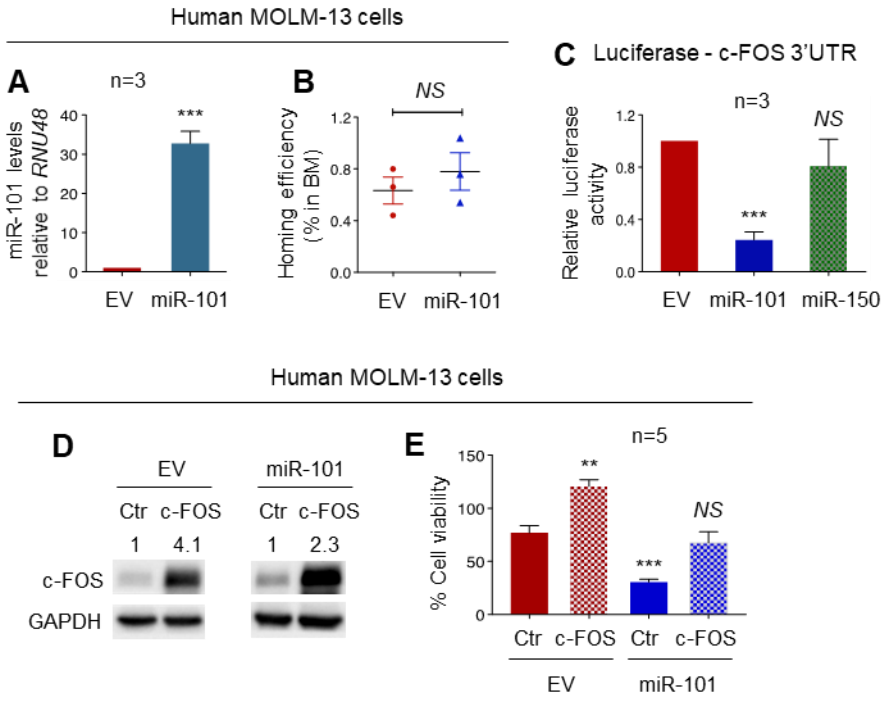
Supplementary Figure 1. (**A**) Heat map representation of microarray analysis showing differential gene expression in MLL-AF9 versus Hoxa9/Meis1 pre-LSCs (n=6) with a cut-off of $P < 0.05$ and FC >1.5. (**B**) RT-qPCR analysis confirming differential expression of genes identified by microarray analysis in MLL-AF9 versus Hoxa9/Meis1 pre-LSCs. (**C**) Heat map representation of Exiqon miRCURY LNA microRNA array analysis showing differential miRNA expression in MLL-AF9 versus Hoxa9/Meis1 pre-LSCs (n=6) with a cut-off of $P < 0.05$ and FC >1.25. (**D** and **E**) RT-qPCR analysis validating miRNA expression in MLL-AF9 versus Hoxa9/Meis1 pre-LSCs (**D**) and in MLL-AF9 LSCs versus normal HSPCs (**E**). Results were normalized using *snoRNA202* as control. Error bars are shown as mean ± SEM of three independent experiments unless specified otherwise. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0005$; ****, $P < 0.0001$.



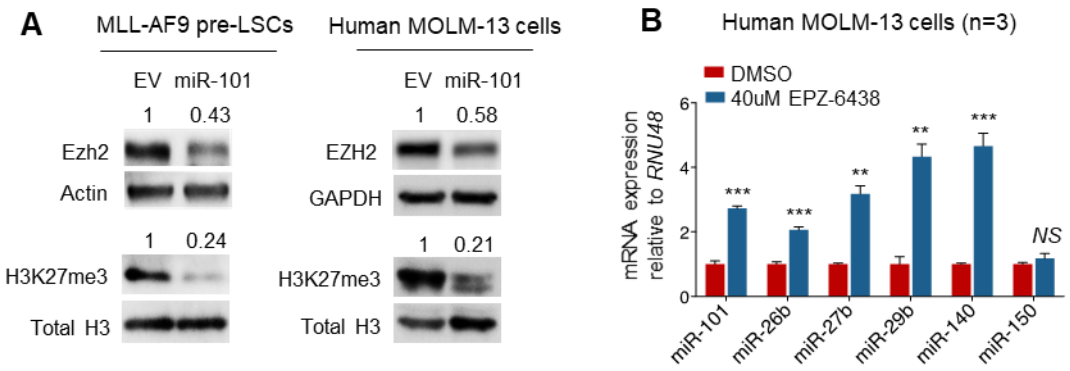
Supplementary Figure 2. (**A**) RT-qPCR analysis confirming miRNA overexpression in MLL-AF9 pre-LSCs transduced with miR-101, miR-150 and miR-29b, respectively. (**B**) Serial colony replating assay of miRNA-expressing MLL-AF9 pre-LSCs. *Note*: The number of colonies produced from week 1 is not shown given cells under selection in the first week post-transduction. (**C**) RT-qPCR confirming miR-101 target gene expression in MLL-AF9 pre-LSCs. (**D**) Total numbers of expanded MLL-AF9 pre-LSCs after a 5-day incubation in methylcellulose supplemented with appropriate cytokines. The cell number per dish at the 6th round of replating is shown (n=3). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0005$; ****, $P < 0.0001$.



Supplementary Figure 3. Representative flow plots depicting changes in the LSC population ($Lin^-CD127^-c\text{-}Kit^+Sca1^-GFP^+CD16/32^{high}CD34^+$) upon miR-101 overexpression in the BM of mice that developed primary MLL-AF9 AML.



Supplementary Figure 4. (**A**) RT-qPCR confirming miR-101 overexpression in transduced human MLL-AF9 (MOLM-13) cells. (**B**) BM homing of human MOLM-13 cells expressing EV versus miR-101, assessed 21 hours after transplantation. $1x10^6$ cells were transplanted into NSG mice (n=3) and human AML cells were identified from mouse BM by staining for human CD45 and FACS analysis. (**C**) Luciferase activity of the c-FOS 3'UTR reporter gene in the absence or presence of miR-101 and miR-150, respectively. miR-150 served as a control miRNA as it has no predicted binding sites in the 3'UTRs of *c-FOS*. HEK293T cells were cotransfected with the reporter constructs for luciferase assays. ***, $P < 0.0005$; *NS*, not significant ($P > 0.05$). (**D**) Western blot analysis confirming enforced overexpression of c-FOS in human MOLM-13 cells expressing EV or miR-101. (**E**) Relative changes in cell viability upon c-FOS overexpression in human MOLM-13 cells expressing EV versus miR-101. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0005$; *NS*, not significant ($P > 0.05$).



Supplementary Figure 5. (**A**) Western blot analysis showing miR-101-induced reduction in levels of EZH2 and H3K27me3 in murine MLL-AF9 pre-LSCs and human MLL-AF9 (MOLM-13) AML cells. (**B**) RT-qPCR analysis of miRNA expression alteration in response to a selective EZH2 inhibitor, EPZ-6438, in human MOLM-13 cells. **, $P < 0.01$; ***, $P < 0.0005$; *NS*, not significant ($P > 0.05$).

Methods

Mice

Six- to 8-week-old C57BL/6 (BL6) and NSG female mice were obtained from Australian BioResources, Mossvale, NSW, Australia. All animal experiments were approved by the Animal Ethics Committee of the University of New South Wales, Sydney, NSW, Australia.

Retroviral constructs and viral transfection

Murine stem cell virus (MSCV)-based retroviral vectors carrying expression cassettes containing *HOXA9*, *MEIS1* and *MLL-AF9* have been described in our previous studies.[1-3] The retroviral MSCV-miR-101-puro, MSCV-miR-29b-puro and MSCV-miR-150-puro expression vectors were generated by GenScript (NJ, USA). pLX304-FOS-V5 was a gift from William Hahn (Addgene plasmid 59140)[4] and pLX304 was a gift from David Root (Addgene plasmid 25890).[5] Viral stocks were produced from 293T cells by transient transfection using Lipofectamine 2000. Virus-containing supernatants were harvested 48-72 hours post-transfection, filtered through 0.45-µm membrane, and concentrated by centrifugation.

Hematopoietic cell isolation, transduction and transplantation

HSPC-enriched LSK cells ($Lin^-CD127^-Sca1^+c\text{-}Kit^+$, BioLegend, Balcatta, WA, Australia) were flow-sorted from mouse BM as previously described.[1,2,6] To generate pre-LSCs, 3-5x10^4 HSPCs were incubated with concentrated virus carrying MLL-AF9 or HOXA9/MEIS1 supplemented with 7.5 µg/ml polybrene (Sigma-Aldrich, Castle Hill, NSW, Australia). Two rounds of transduction were performed for each experiment. For mouse bone marrow transplantation, 1x10^6 GFP^+ pre-LSCs or 1x10^5 GFP^+ leukemic cells flow-sorted from primary AML mice were injected intravenously by the tail vein into sublethally irradiated (6 Gy) BL6 syngeneic recipient mice as previously described.[1-3]

Fluorescence-activated cell sorting (FACS)

Alexa Fluor 647 anti-human CD45 antibodies were purchased from BioLegend (304018). Cells were stained with antibodies at 4 °C for 20 min before being subjected to FACS analysis using a BD LSRFortessa™ cell analyzer (BD Biosciences, North Ryde, NSW, Australia). Cell sorting was conducted using a BD Influx™ high-speed cell sorter (BD Biosciences). Flow cytometry data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

In vitro cell cycle and apoptosis

For cell cycle analysis, cells were fixed in ice-cold 70% ethanol for 2 hours at -20 °C, stained with anti-Ki67-Alexa Fluor 647 (BD Biosciences) and 7-amino-actinomycin D (7AAD, BD Biosciences) in staining solution (50 µg/ml propidium iodide, 0.1% Triton X-100 and 100 µg/ml DNase-free RNaseA) and incubated for 20 min at 4 °C prior to flow cytometric analysis. For apoptosis analysis, cells were incubated with Annexin V-APC staining solution (BD Biosciences, 550474) at room temperature for 10 minutes, followed by staining with 7-Aminoactinomycin D (7-AAD) (BD Biosciences, 559925) for 15 min on ice and subsequent analysis by flow cytometry.

miRNA and mRNA gene expression profiling

Total mRNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany, 74106) and miRNA was extracted using the miRNeasy mini kit (Qiagen, 217004). The array data are available in ArrayExpress under the accession numbers E-MTAB-7344, E-MTAB-7355 and E-MTAB-7356.

Labelling, hybridization and scanning were performed by the Ramaciotti Centre for Gene Function Analysis (University of New South Wales, Sydney, Australia), with mRNA hybridized to Illumina murine WG-6 v2.0 expression array for mRNA expression profiling and miRNA hybridized to Exiqon miRCURY LNA microRNA Array for global microRNA expression profiling. The mRNA expression profiling was analyzed using the GenePattern software (Broad Institute, MIT) as previously described.[2]

Bayesian Networks with Splitting-Averaging strategy used to identify miRNA-mRNA network
A method called Bayesian network with splitting-averaging (BNSA) was used to identify complex miRNA-mRNA interactions for functional miRNA-mRNA regulatory networks (FMRMs). This method integrates miRNA-target information, sample-matched expression profiles of miRNA and mRNA, and sample categories. In order to capture all possible interactions, this method groups expression profiles of miRNAs and mRNAs together according to their sample category and then learns Bayesian Network (BN) structures on the relative expression changes of miRNA and mRNA in each category, respectively. The miRNA-target information acts as a constraint to guide the structure learning, whereby the miRNAs represent the parent nodes while the mRNAs are the descendant nodes. The edges linking the parent nodes to descendant nodes can only be those defined in the miRNA-target predictions. Interaction networks learned on each category are then integrated by BN averaging procedure. To avoid statistically insignificant results due to the small size of data sets, it uses bootstrapping to achieve reliable inference and integration.[7]

Cell viability assay
Cell viability was measured by the AlamarBlue™ cell viability assay (Thermo Fisher Scientific, North Ryde, NSW, Australia, DAL1025) as per manufacturer's instruction. Briefly, $5x10^3$ human MOLM-13 cells were seeded in 96-well plates with 180 µL media per well. After 48 hours 20 µL of AlamarBlue reagent was added into each well and incubated at 37°C for 6 hours before absorbance values were measured at 570 and 595 nm using the Benchmark Plus microplate reader (Bio-Rad, Gladeville, NSW, Australia).

Colony formation assay
Cells were seeded at a density of $1x10^3$ cells in 35 mm dishes in methylcellulose supplemented with mIL-3. Colonies were counted after 5 days incubation at 37°C. For serial replating assay, colonies were harvested, and $1x10^3$ cells were subsequently replated in fresh methylcellulose. Six rounds of serial replating were performed.

Real-time quantitative qPCR analysis (RT-qPCR) for gene expression
Total RNA was extracted from cells using the RNeasy mini kit (Qiagen, 74106). cDNA was synthesized from total RNA using random primers (Thermo Fisher Scientific, 48190011) and M-MLV Reverse Transcriptase (Thermo Fisher Scientific, 28025013). RT-qPCR was performed using the Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific, 436708) or SsoAdvanced Universal SYBR Green Supermix (Bio-Rad).

The values for target gene expression were normalized against the house-keeping gene Gapdh. RT-qPCR primers include mouse Bcl11a: forward 5'-GCGACACTTGTCCTTCACACACC-3', reverse 5'-GCTTCCATCCGAAAACTGCCACAC-3'; Mouse Meis2: forward 5'-GCAATCTATGGGCAC CCGTTGT-3', reverse 5'-CGGCGCGAACCTGCTTG-3'; mouse Six1: forward 5'-CAAGGAAAG

GGAGAACACCGA-3', reverse 5'-TGGAGCAGAAGGACCGAGT-3'; mouse Sall3 forward 5'-CACCCAAGCAGCACAACTGTC-3', reverse 5'-TGCCCATGTGTACCTTGAGATTGC-3'; mouse Tcf7l2: forward 5'- TTCCTCCGATCACAGACCTGAG-3' and reverse: 5'-GCTGCCTTCACCTTGTATGTAGC-3'; mouse p21: forward 5'-TTGCACTCTGGTGTCTGAGC-3' and reverse 5'- TGCGCTTGGAGTGATAGAAA-3'; mouse Gapdh: forward 5'-CTTTGTCAAGCTCATTTCCTGG-3' and reverse 5'-TCTTGCTCAGTGTCCTTGC-3'.

RT-qPCR analysis for miRNAs

miRNAs were extracted from cells using the miRNeasy mini kit (Qiagen, 217004). cDNA was synthesized using a Taqman microRNA reverse transcription (RT) kit (Life Technologies, Mulgrave, VIC, Australia, 4366596) and RT primers (Taqman microRNA assay 5X; Life Technologies, 4427975; see below for Assay details) specific for a target miRNA or a house-keeping miRNA. RT-qPCR was performed using the Taqman Universal PCR Mastermix II (2X), no UNG (Life Technologies, 4324018) and specific miRNA probes (Taqman microRNA assay 20X; see below for Assay details). The values for mouse and human miRNAs were normalized against their house-keeping genes, mouse snoRNA202 and human RNU48, respectively.

RT-qPCR primers for human and mouse miRNAs (Taqman)

	Assay Name	Assay ID
House-keeping miRNA for murine miRNAs	snoRNA202	001232
miR-101	mmu-miR-101	002507
miR-29a	mmu-miR-29a	002447
miR-29b	mmu-miR-29b	002497
miR-222	mmu-pri-miR-222	Mm03307187_pri
miR-150	mmu-miR-150	000473
House-keeping miRNA for human miRNAs	RNU48	001006
miR-101	hsa-miR-101	002253
miR-29a	hsa-miR-29a	002112
miR-29b	hsa-miR-29b	000413
miR-26b	hsa-miR-26b	000407
miR-27b	hsa-miR-27b	000409
miR-140	hsa-miR-140	002234
miR-222	hsa-miR-222	002276
miR-150	hsa-miR-150	000473

Western blot analysis

Membranes were probed with antibodies against Meis1 (Abcam, Melbourne, VIC, Australia, ab124686), Mcl-1 (Abcam, ab32087), Bcl-2 (Genesearch, Arundel, QLD, Australia, 3498S), p21 (BD Biosciences, 556431), p27 (BD Biosciences, 610241), Gadd45a (Genesearch, 4632S), c-Fos (Genesearch, 4384S), phospho-c-Fos (Ser32; Genesearch, 5348S), Ezh2 (Genesearch, 3147S), H3K27me3 (Genesearch, 9733T), total H3 (Genesearch, 9715S), Actin (Sigma-Aldrich, A2066) and GAPDH (Abcam, ab8245). Densitometry was performed using NIH ImageJ software. Protein band intensity was normalized against loading control (Actin, GAPDH or H3) and compared to relative control.

Confocal immunofluorescence

4 x 10^4 cells were cytospun onto glass slides, fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature and then washed three times for 5 minutes in PBS with 0.1% Tween-20 (PBS-T). Non-specific antibody binding was blocked with blocking media (10% goat serum and 2% BSA in PBS-T) for 1 hour. Slides were stained overnight at 4C with a mouse monoclonal antibody against non-phospho (active) β-catenin (Ser33/Ser37/Thr41; clone 8E7, Merck, Bayswater, VIC, Australia, 05-665) at a dilution of 1:100, washed three times for 5 minutes in PBS-T, and then stained for 1 hour with Alexa Fluor 568-conjugated donkey anti-mouse secondary antibody (Abcam, ab175472) at a dilution of 1:500 and washed three times with PBS-T for 5 minutes followed by staining with DAPI (1:4000, Thermo Fisher Scientific) for 30 minutes. Coverslips were mounted with ProLong Gold antifade mountant (Thermo Fisher Scientific) and edges were sealed with nail polish to prevent desiccation. Immunofluorescence images were taken with a TCS SP5 MP-STED confocal microscope (Leica Microsystems, Mannheim, Germany) and processed using NIH ImageJ software.

***In vivo* bioluminescence imaging of NSG mice**

GFP-luciferase-expressing human MOLM-13 AML cells were generated by transducing retroviral vector SFG-NES-TGL (GFP-FLuc; a gift from Vladimir Ponomarev)[8] into MOLM-13 cells (ATCC, Noble Park North, VIC, Australia; recently authenticated by Short Tandem Repeat PCR and tested for mycoplasma contamination) followed by flow-sorting of GFP^+ transduced cells. 5 x 10^5 transduced cells were injected intravenously by the tail vein into NSG mice, which were monitored for assessing *in vivo* engraftment of human AML cells using non-invasive bioluminescent imaging. Images were taken 10 min after intraperitoneal injection of D-luciferin (150 mg/kg of body weight; Promega, P1043) for FLuc signals detected by the IVIS Spectrum CT (Caliper Life Sciences, MA, USA).[8,9] Signal intensity quantification and analysis were performed using the Living Image 4.5.5 software (Perkin Elmer, Waltham, MA, USA). The bioluminescent signal was recorded as radiance photons/sec/cm^2/steradian (p/s/cm^2), represented in pseudocolor to indicate the signal intensities.

Luciferase Reporter Assay

For transfection, 6x10^3 293T cells/well were plated in 96-well plates in triplicate for each condition. After incubation overnight, cells were transfected with 20 ng of the pMIR-REPORT plasmid (pMIR-REPORT miRNA expression vector system; Thermo Fisher Scientific, AM5795) bearing the 3'UTR of *c-FOS*, and 20 ng of MSCV-miR-101-puro or MSCV-miR-150-puro versus control empty vector (EV) using Lipofectamine 3000 (Thermo Fisher Scientific, L3000015). pMIR-REPORT™ Beta-galactosidase Reporter Control Vector (Thermo Fisher Scientific, AM5795) (1 ng) was cotransfected for transfection efficiency control in all transfections. Transduced cells were lysed, and firefly luciferase and β-galactosidase activities were detected using Dual-Light® Luciferase & β-Galactosidase Reporter Gene Assay System (Thermo Fisher Scientific, T1003/T1004) 48h post-transfection following the manufacturer's protocol. Firefly luciferase activity was normalized to β-galactosidase activity for each transfected well. Each experiment was performed in triplicate and repeated three times.

Statistical analysis

Statistical significance of differences was determined by an unpaired two-tailed Student's *t*-test for comparison between two groups and log-rank test for Kaplan-Meier survival curves using GraphPad Prism 7 (La Jolla, CA, USA). Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, ****$P < 0.0001$, *NS* = Not Significant.

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