ARTICLE - Acute Myeloid Leukemia

miR-30e-5p regulates leukemia stem cell self-renewal through the Cyb561/ROS signaling pathway

Yanwen Ge,[1] Mei Hong,[1] Yu Zhang,[1] Jiachen Wang,[1] Lei Li,[2] Hongkai Zhu,[3] Yue Sheng,[3] Wen-Shu Wu[4] and Zhonghui Zhang[1,5]

[1]School of Life Sciences, Shanghai University, Shanghai, China; [2]Department of Pediatrics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China; [3]Department of Hematology, The Second Xiangya Hospital, Central South University, Changsha, China; [4]Division of Hematology/Oncology, Department of Medicine and University of Illinois Cancer Center, the University of Illinois at Chicago, Chicago, IL, USA and [5]Shaoxing Institute of Technology, Shanghai University, Shaoxing, China

Correspondence: Z. Zhang
zhonghui@shu.edu.cn

W-S. Wu
wuwenshu@uic.edu

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Supplementary Methods

Retroviral and lentiviral transduction of hematopoietic cells and AML cells

For preparing shRNA lentiviruses, 293T cells were transfected with a DNA mixture containing 2.5 μg of shRNA lentiviral vectors and 2.5 μg of the packaging mixture containing pMD2.G and psPAXs (Addgene) by Lipofectamine™ 2000. For generation of retroviral particles, 293T cells were transfected with a DNA mixture containing 1.5 μg of pCL-Eco (IMGENEX), 2.5 μg of retroviral vector only, or retroviral expression vectors by Lipofectamine™ 2000 (ThermoFisher Scientific) according to the manufacturer's instructions. All media containing retroviral or lentiviral particles were collected 24 hours after transfection and filtered through a 0.45 μm pore-size filter.

To transduce HSPCs, cells were enriched from BM of mice following 5-FU injection (150 mg/kg body weight). Enriched HSPCs or AML cells were cultured overnight and were centrifuged at 200 X g. The supernatant was aspirated and replaced with retroviral- or lentiviral particle-containing supernatant supplemented with 5 μg/ml polybrene (Sigma) followed by centrifugation (900 X g for 45 min). After two days of transduction, the transduced cells were selected with 1 μg/ml puromycin and/or 10 μg/ml blasticidin, and then were used for the further experiments.

Patient samples and AML cell lines

The healthy bone marrow and primary AML patient samples were obtained from Union Hospital (Wuhan, China), under a protocol approved by the Ethical Committee on Medical Research at Tongji Medical College. Research was conducted in accordance with the Declaration of Helsinki, with patient informed consent. Human AML cell lines were purchased from National Collection of Authenticated Cell Cultures (Shanghai, China).

BM transplantation and NAC/Doxycycline/miRNA agomir administration

8-10-week-old C57BL/6J mice received acidified antibiotic water containing Sulfamethoxazole and Trimethoprim for one week, and then lethally or sublethally irradiated before transplantation. Transduced HSCs and LSCs were transplanted into mice by retro-orbital injection. Recipient mice were continuously fed with acidified antibiotic water for three weeks. For secondary transplantation, cells from the primary recipients were injected into sublethally-irradiated secondary recipients.

For NAC treatment, recipient mice were injected intraperitoneally (i.p.) once daily with NAC (Sigma-Aldrich) (100 mg/kg body weight) or PBS (control group) for ten consecutive days.

For doxycycline administration, recipients were fed the sterilized water containing 1 mg/ml doxycycline (Sigma).

For *in vivo* miRNA agomir treatment, the recipient mice were injected intraperitoneally with 5 nmol of synthetic miR-30e-5p agomir or control agomir in 100 μl of saline twice per week for 3 weeks. The synthetic control agomir and miR-30e-5p agomir (2'OME+3'chol modified) were purchased from Sangon (Shanghai, China). The sequences of control agomir and miR-30e-5p agomir were listed in Supplementary Table 10.

Western blot analysis

For preparing protein samples, the AML cells were lysed by RIPA buffer containing proteinase and phosphatase inhibitors from Sangon (Shanghai, China). Western blotting was performed using standard procedures. The anti-CYB561 antibody (PA5103405) was from ThermoFisher Scientific (Shanghai, China).

Intracellular glutathione, ascorbate and dehydroascorbate detection

The intracellular levels of glutathione, ascorbate and dehydroascorbate were measured by the kits from Sangon (Shanghai, China) according to the manufacturer's instructions. Briefly, the

AML cells were performed freeze-thaw process. The supernatants were collected by centrifugation at 12000 *g* for 5 min at 4°C and performed for the further measurement.

Transfection of AML cells with miRNA and siRNA

Synthetic miR-30e-5p mimic and miRNA negative control were purchased from RIBOBIO (Guangzhou, China). The sequences of miRNA negative control and miR-30e-5p mimic were listed in Supplementary Table 10. CYB561 siRNA and siRNA negative control were purchased from RIBOBIO (Guangzhou, China). The mouse/human CYB561 siRNA target sequence was as follows:5'-ACTGGAAGCGGCCTTCCCA-3'. All miRNAs or siRNAs were dissolved in distilled H_2O. $1x10^5$ mouse and human AML cells were transfected with miRNAs or siRNAs at a final concentration of 100 nmol/l by the ribo*FECT*^{TM} CP Reagent from RIBOBIO (Guangzhou, China) according to the manufacturer's instructions. After 24 hours of transfection, the transfected cells were further performed the proliferation assay.

Colony-forming assay

Transduced HSPCs were plated in methylcellulose (R&D systems) with 50 ng/ml murine SCF, 10 ng/ml murine IL-3, and 10 ng/ml murine IL-6, and counted and passaged after 5-7 days. Values represent colony-forming unit frequencies, calculated as number of colonies per input cells (%).

Homing assay for LSCs

The recipient mice were lethally irradiated (9 Gy), and then injected $1x10^6$ AML cells each. After 16 hours of transplantation. The BM cells were isolated from the recipients and directly analyzed for the ratio of GFP-positive cells in total BMMNCs by flow cytometry.

Peripheral blood analysis

Blood samples were collected into EDTA-coated tubes via lateral tail vein incision and analyzed on a Hemavet 950 analyzer (Drew Scientific). PB smears were stained with Wright-Giemsa staining according to the manufacturer's instructions (BioScientific).

Flow cytometric analysis

BM mononuclear cells (MNCs) were isolated from mouse BM and purified by density gradient centrifugation through Ficoll. For analysis of $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) cells, one million of BM MNCs were stained with a mixture of biotinylated antibodies against mouse CD11b, CD3e, CD45R (B220), Ly-6G (Gr-1), and TER-119. Subsequently, the cells were co-stained with streptavidin-Prep-Cy5.5, anti-Sca-1-APC, and anti-c-Kit-PE/Cy7. For sorting and analyzing HSC cells, BM MNCs were co-stained with anti-CD135-PE and anti-CD34-FITC. For analysis of LSCs, one million BM MNCs or splenocytes were stained with a mixture of biotinylated antibodies and then co-stained with streptavidin-Prep-Cy5.5, anti-c-Kit-PE/Cy7, anti-CD34-APC, and anti-CD16/32-APC/Cy7. For cell-cycle analysis, cells were stained with Hoechst 33342 and Pyronin Y (Sigma) in HBSS containing 10% fetal bovine serum. For cell cycle analysis, cells were stained with Hoechst 33342 and Pyronin Y, then co-stained with the antibodies described above. For apoptosis analysis, cells were stained with Annexin V in Annexin V buffer according to the manufacturer's instructions. For intracellular ROS detection, cells were stained with fluorometric ROS dye (Sigma, China) for 30 min at 37 °C and co-stained with LSC antibodies. All antibodies, unless otherwise stated, were purchased from Biolegend and ThermoFisher Scientific (Supplementary Table 11). Flow cytometry was performed on a CytoFLEX or MoFlo XDP (Beckman Coulter), and all flow cytometric data were analyzed with FlowJo Software (TreeStar).

RNA extraction, reverse transcription-PCR, and Real-time PCR

Total RNA samples were extracted using Quick-RNA MicroPrep Kit (Zymo Research). For real-time PCR, 100 ng of total RNA was reverse transcribed using Superscript III™ cDNA Synthesis Kit (Invitrogen). *Hprt* or *Gapdh* expression were used as internal controls to normalize relative expression of each gene. For detecting miRNA expression, total RNAs were reverse-transcribed using miDETEECT A Track miRNA qRT-PCR Kit from RIBOBIO (Guangzhou, China). The list of primers is included in the Supplementary Table 1.

Luciferase reporter assay

293T cells were seeded in 24-well plates overnight, and then co-transfected with 50 ng of the luciferase reporters, 925 ng of miRVector (pMXs-miR-Puro) or pMXs-miR-30e-Puro, and 25 ng of pCMV-LacZ using Lipofectamine™ 2000. After 72 hours of transfection, cells were lysed in 250 µl of the passive lysis buffer (Promega) and assayed with a luciferase assay kit (Promega) as directed by the manufacturer. Luciferase activities were expressed as relative luciferase/LacZ activities and normalized to those of control transfections in each experiment.

Limiting dilution assays

LSCs isolated from BM of the recipient mice that developed full-blown leukemia were co-stained with LSC antibodies, sorted, and injected into sublethally irradiated C57BL/6J mice with three different doses of donor cells for each group. The number of recipient mice developed leukemia was counted for each group with each dose of donor cells. ELDA software was used to estimate the frequency of LSCs/LICs.

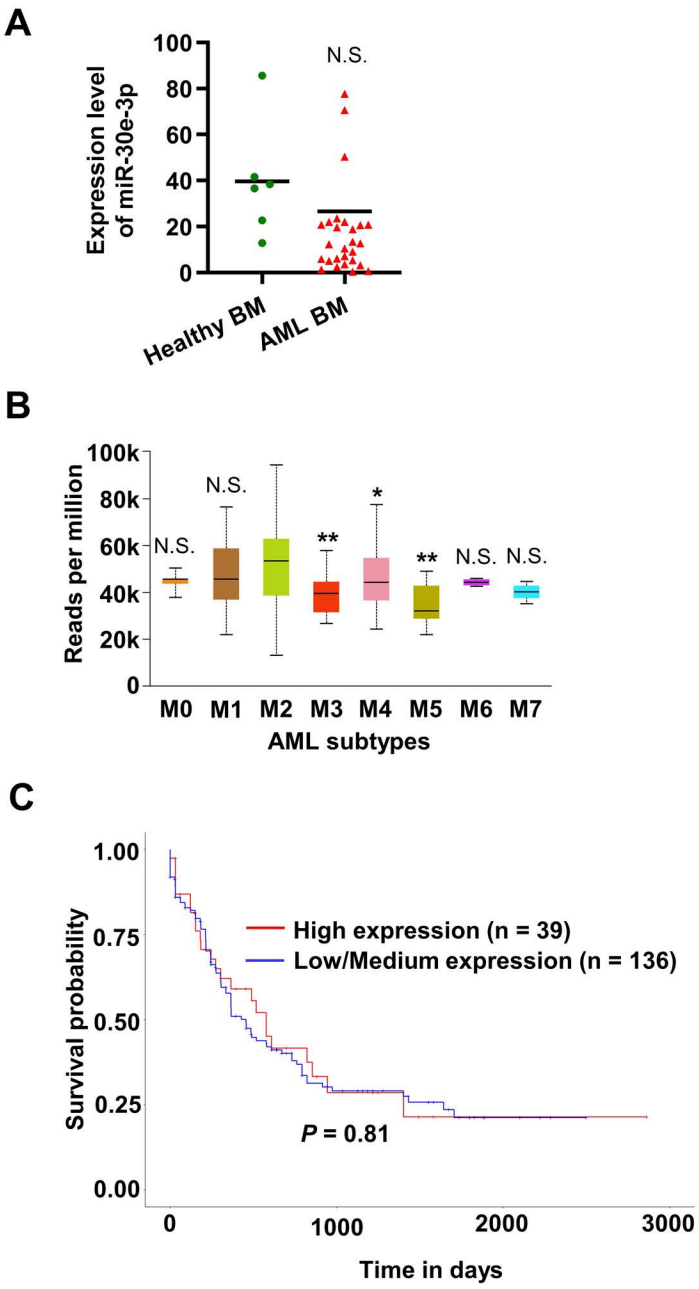
RNA sequencing and analysis

LSCs were sorted by flow cytometry and then performed RNA extraction. The LSC cDNA library was prepared using a SMART-Seq HT Kit (TAKARA, Mountain View, USA), and then sequenced on an Illumina NovaSeq 6000 Sequencing System (Illumina, San Diego, USA) in the

150 bp paired-end mode. Library construction, sequencing and data analysis were performed by Shanghai Sinomics Corporation (Shanghai, China). Differentially expressed genes were compared in miRVector LSCs and miR-30e-5p-overexpressed LSCs, and those with a fold change > 2.0, and $P < 0.05$ were selected as differentially expressed genes for further analysis. KEGG pathway analysis was performed using the enrich R package (https://www.genome.jp/kegg/). Gene set enrichment analysis (GSEA) was performed with GSEA v4.3.2 software, available from the Broad Institute of Massachusetts Institute of Technology (http://www.gsea-msigdb.org/gsea/index.jsp). When performing GSEA analysis, m2.cp.wikipathways.v2023.1.Mm.symbols.gmt containing a collection of 133 gene sets of the interest. Following parameters were used: Number of permutations = 1000, permutation type = gene_set. Other parameters were left at default values.

Statistical analyses

Unpaired Student's t-test was used to statistically analyze all the two experimental groups. A log-rank (Mantel-Cox) test was used to determine p values for all Kaplan-Meier survival analyses. Differential expression analysis from RNA-seq data were performed using R package (version 3.4.3) edge R. Differences of $P < 0.05$ were considered statistically significant and are denoted as * $P < 0.05$; ** $P < 0.01$. All qPCR and PCR results were repeated at least three times.

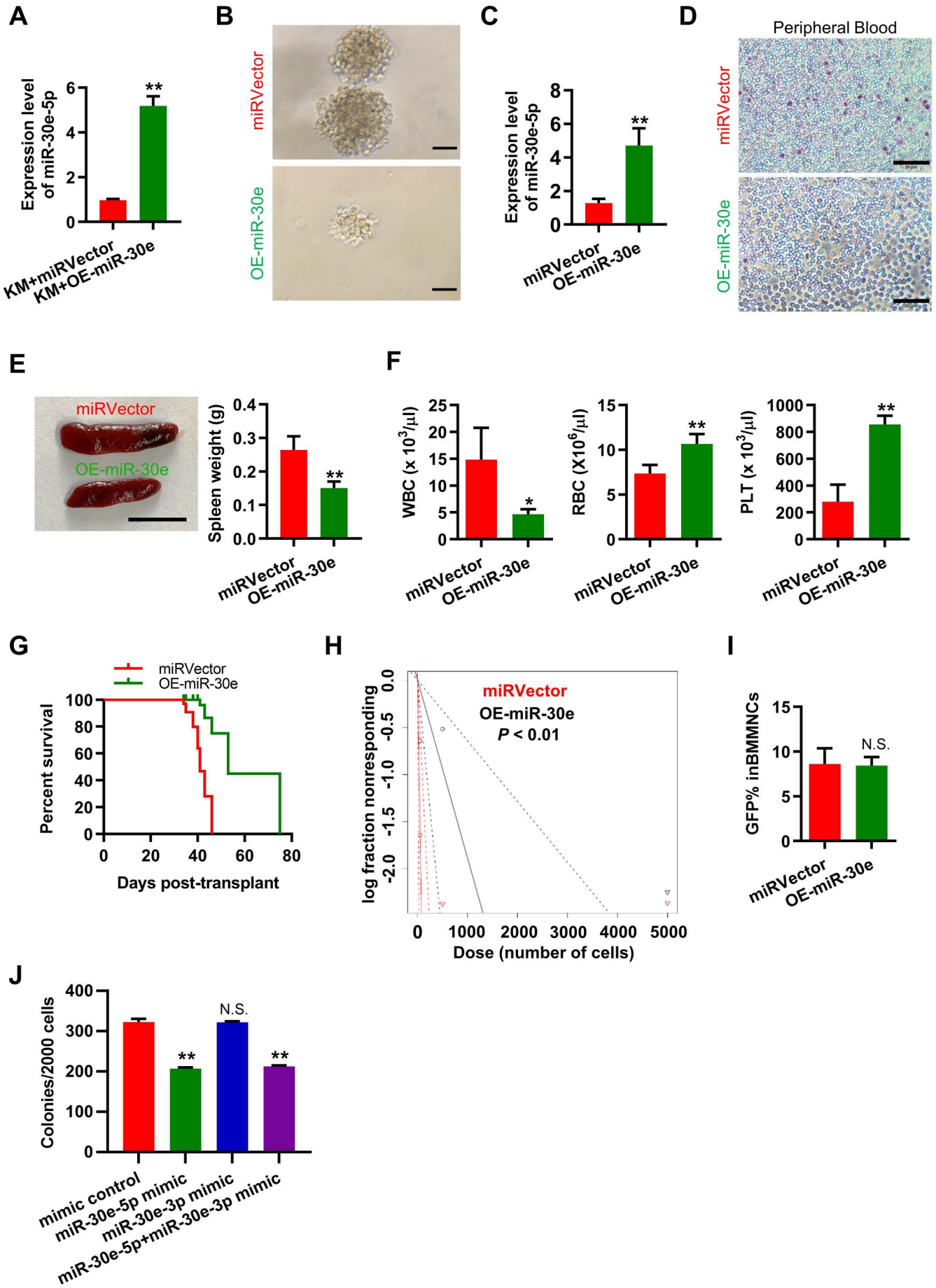


Supplementary Figure 1. Expression levels of miR-30e-5p and miR-30e-3p in AML patients.

(A) qPCR analysis of the expression of miR-30e-3p between bone marrow cells of healthy controls and AML patients. Results are normalized to *U6* expression and expressed relative to miR-30e-3p expression in healthy group (healthy patient, n = 6; AML patient, n = 29).

(B) The expression of miR-30e-5p between different FAB subtypes of AML patients. The data were analyzed by online software using TCGA database (http://ualcan.path.uab.edu/index.html) (*$P < 0.05$; ** $P < 0.01$; N.S., no significance).

(C) The analysis of prognostic significance of miR-30e-5p in AML patients. The data were analyzed by online software using TCGA database (http://ualcan.path.uab.edu/index.html).


A
Expression level of miR-30e-5p
KM+miRVector
KM+OE-miR-30e
B
miRVector
OE-miR-30e
C
Expression level of miR-30e-5p
miRVector
OE-miR-30e
D
Peripheral Blood
miRVector
OE-miR-30e
E
miRVector
OE-miR-30e
Spleen weight (g)
F
WBC (x 10^3/μl)
RBC (X10^6/μl)
PLT (x 10^3/μl)
G
miRVector
OE-miR-30e
Percent survival
Days post-transplant
H
miRVector
OE-miR-30e
P < 0.01
log fraction nonresponding
Dose (number of cells)
I
GFP% inBMMNCs
N.S.
J
Colonies/2000 cells
N.S.
mimic control
miR-30e-5p mimic
miR-30e-3p mimic
miR-30e-5p+miR-30e-3p mimic

Supplementary Figure 2. Phenotype analysis in KMT2A::MLLT3 derived AML mice.

(A) qPCR analysis of the expression of miR-30e-5p in HSPCs co-transduced with miRVector/KMT2A::MLLT3 (KM) or OE-miR-30e/KMT2A::MLLT3 (KM). Results are normalized to *U6* expression and expressed relative to miR-30e-5p expression in OE-miR-30e/KMT2A::MLLT3 group (n = 3).

(B) Colony morphology of miRVector- or miR-30e-overexpressed AML cells at passage 2 (scale bar = 50 μm).

(C) qPCR analysis of the expression of miR-30e-5p in AML cells from primary recipients. Results are normalized to *U6* expression and expressed relative to miR-30e-5p expression in OE-miR-30e group (n = 3).

(D) Representative peripheral blood smear stained by Wright-Giemsa (scale bar = 50 μm).

(E) Spleen morphology (left, scale bar = 1 cm) and weight (right) from secondary recipients injected with miRVector- or miR-30e-overexpressed AML cells at week 3 post-transplantation (n = 5).

(F) Complete blood count (CBC) analysis of peripheral blood in the primary recipients injected with miRVector- or miR-30e-overexpressed AML cells at 46 days post transplantation (n = 4).

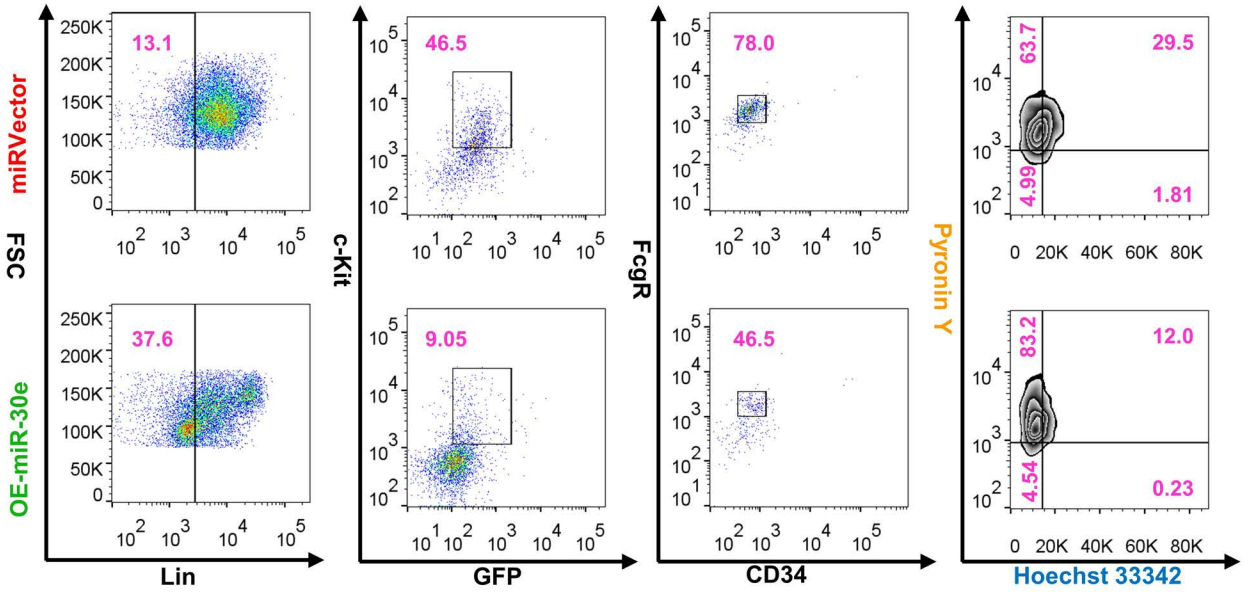
(G) Survival analysis of secondary recipient mice. Media survival was 41 versus 53 days posttransplant for primary recipients of miRVector- or miR-30e-overexpressed AML cells, respectively ($P < 0.01$, Mante-Cox test, n = 5).

(H) Limiting dilution assay of miRVector- and miR-30e-overexpressed LSCs from secondary transplantation recipients. LSC/LIC frequencies calculated by ELDA software (n= 5). The Chi-squared test was used (** $P < 0.01$).

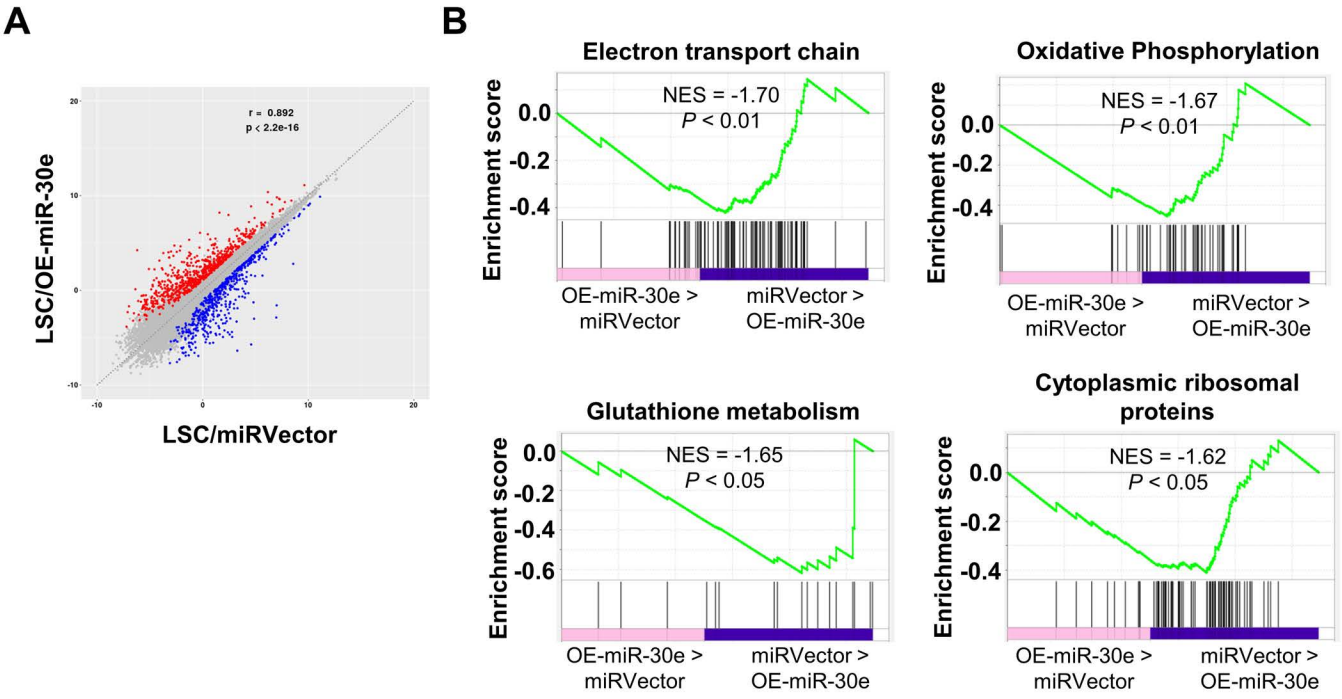
(I) Quantification of homing AML cells at 16 h after transplantation of GFP^+ gated AML cells from secondary recipients (n = 5).

(J) Effects of miR-30e-5p and miR-30e-3p mimics on the colony formation of mouse KMT2A::MLLT3. The mouse AML cells ($1x10^5$) were transfected with synthetic miRNA mimic control, miR-30e-5p mimic, and/or miR-30e-3p (100 nmol/L). After 24 hours of transfection, the transfected cell were performed the colony formation assay (n = 3).

Data are representative of two or three independent experiments. All data are represented as mean ± SD. Two-tailed Student's *t*-tests were used to assess statistical significance (*$P < 0.05$; ** $P < 0.01$; N.S., no significance).



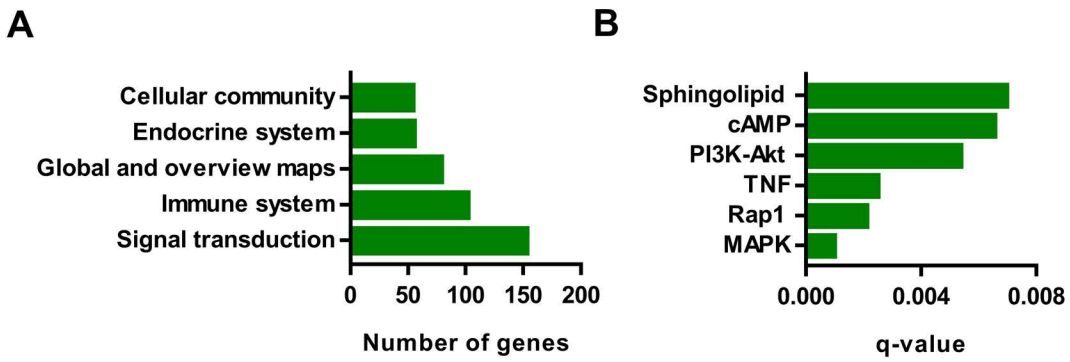
Supplementary Figure 3. Representative flow cytometry analysis of L-GMP in the BM. BM were isolated from primary recipients injected with $5x10^4$ of miRVector- or miR-30e-overexpressed AML cells at week 5 post-transplantation. The L-GMP ($Lin^{-/low}cKit^{+}CD34^{+}CD16/32^{+}$) were analyzed by flow cytometry.



Supplementary Figure 4. Molecular signaling pathways underlying the regulation of miR-30e on LSCs.

(A) Scatter plot showing the differentially expressed genes in miRVector- and miR-30e-overexpressed L-GMPs. The L-GMP cells were sorted from the secondary recipients transplanted with miRVector- or miR-30e-overexpresed AML cells (n = 2). The date were analyzed by R package (version 3.4.3) edgeR.

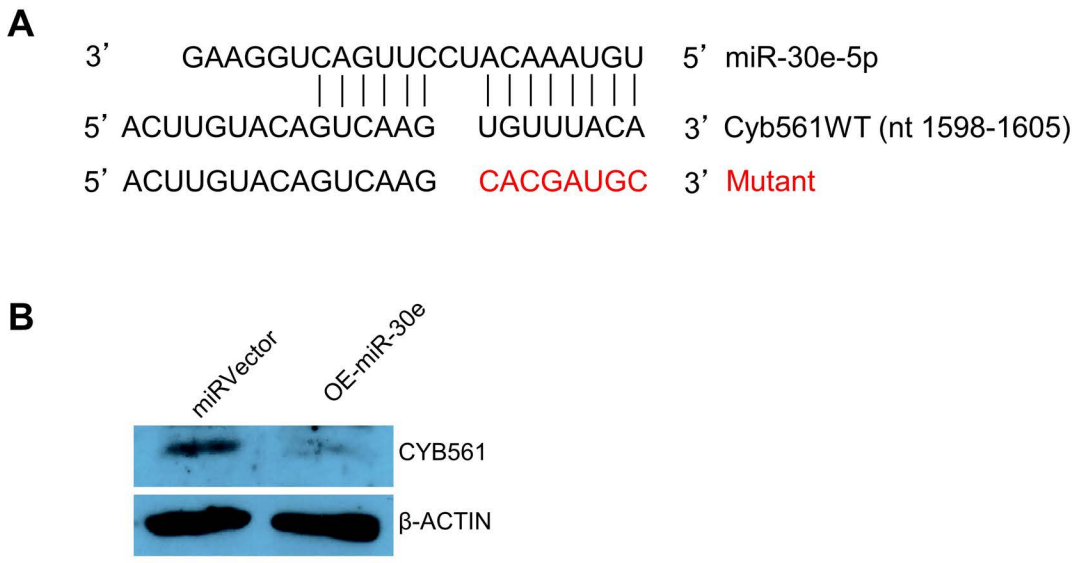
(B) Enrichment plots of downregulated gene sets from GSEA analysis.



Supplementary Figure 5. KEGG Pathway analysis of differentially expressed genes of miRVector- and miR-30e-overexpressed LSCs.

(A) KEGG class analysis of upregulated and downregulated genes ranked by gene number.

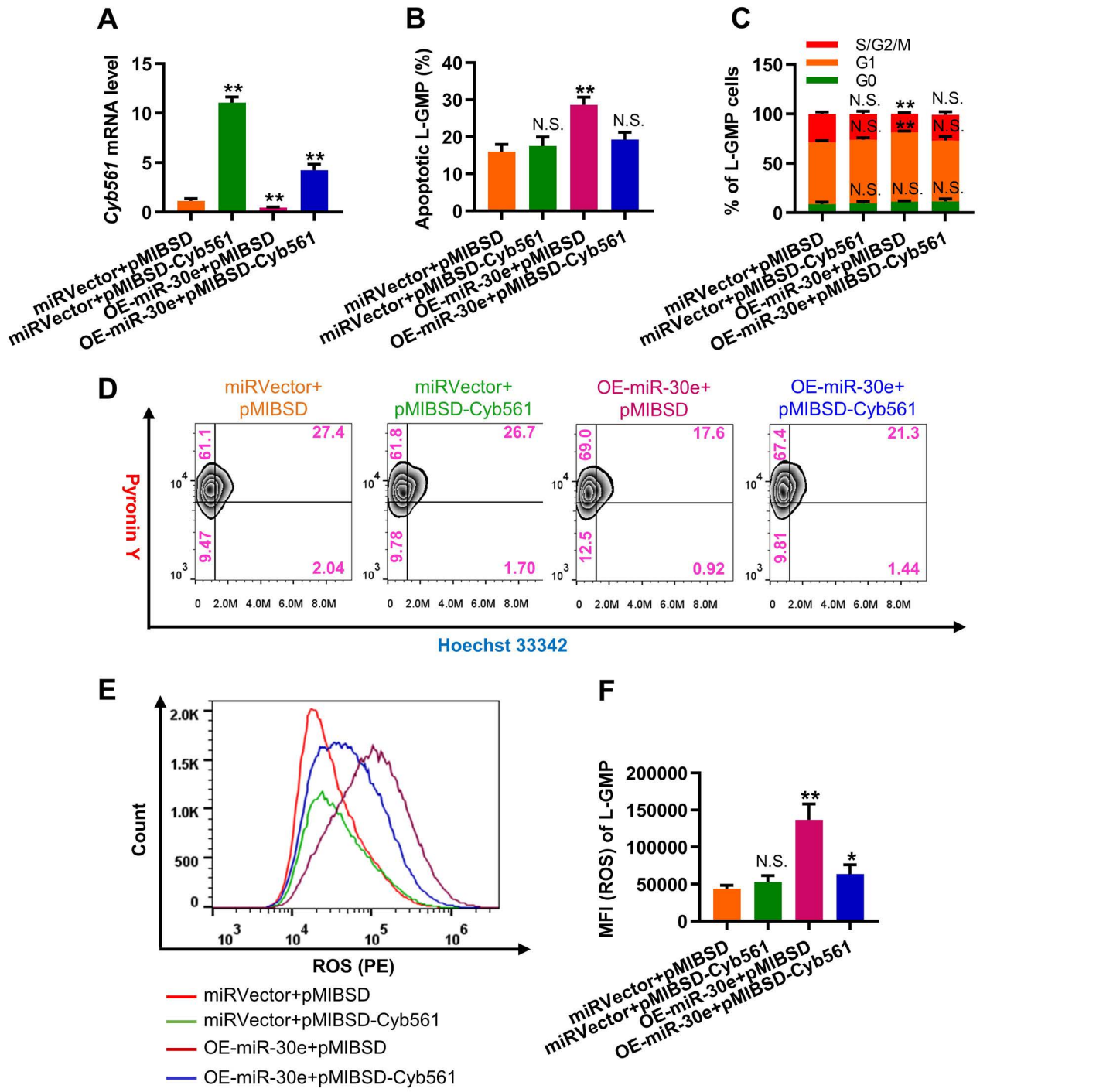
(B) Top signaling pathways in signal transduction of KEGG class ranked by q-value.



Supplementary Figure 6. miR-30e downregulates the endogenous *Cyb561* in AML cells.

(A) The potential binding sequence of the miR-30e-5p in *Cyb561* 3’UTR. The potential binding site of the miR-30e-5p in *Cyb561* were aligned with conserved binding site of the miR-30e-5p. Multiple point mutations (in red) were introduced to generate the mutant *Cyb561* 3’ UTR.

(B) Western blotting analysis of protein level of CYB561 in mouse AML cells. The AML cells were isolated from recipients transplanted with miRVector- or miR-30e-overexpressed AML cells and then performed western analysis.


A
Cyb561 mRNA level
15
10
5
0
**
**
**
miRVector+pMIBSD
miRVector+pMIBSD-Cyb561
OE-miR-30e+pMIBSD
OE-miR-30e+pMIBSD-Cyb561
B
Apoptotic L-GMP (%)
40
30
20
10
0
N.S.
**
N.S.
C
% of L-GMP cells
150
100
50
0
S/G2/M
G1
G0
N.S.
N.S.
**
**
N.S.
N.S.
N.S.
N.S.
N.S.
D
miRVector+ pMIBSD
miRVector+ pMIBSD-Cyb561
OE-miR-30e+ pMIBSD
OE-miR-30e+ pMIBSD-Cyb561
Pyronin Y
61.1
27.4
9.47
2.04
61.8
26.7
9.78
1.70
69.0
17.6
12.5
0.92
67.4
21.3
9.81
1.44
0
2.0M
4.0M
6.0M
8.0M
Hoechst 33342
E
Count
2.0K
1.5K
1.0K
500
0
ROS (PE)
miRVector+pMIBSD
miRVector+pMIBSD-Cyb561
OE-miR-30e+pMIBSD
OE-miR-30e+pMIBSD-Cyb561
F
MFI (ROS) of L-GMP
200000
150000
100000
50000
0
N.S.
**
*
miRVector+pMIBSD
miRVector+pMIBSD-Cyb561
OE-miR-30e+pMIBSD
OE-miR-30e+pMIBSD-Cyb561

Supplementary Figure 7. Effects of miR-30e on the functions of LSC with or without *Cyb561* overexpression.

(A) qPCR analysis of the expression of *Cyb561* in AML cells. Results are normalized to *Hprt* expression and expressed relative to miRVector/pMIBSD-transduced AML cells (n = 3).

(B) Percentage of apoptotic LSCs in the BM from secondary recipients (n = 4).

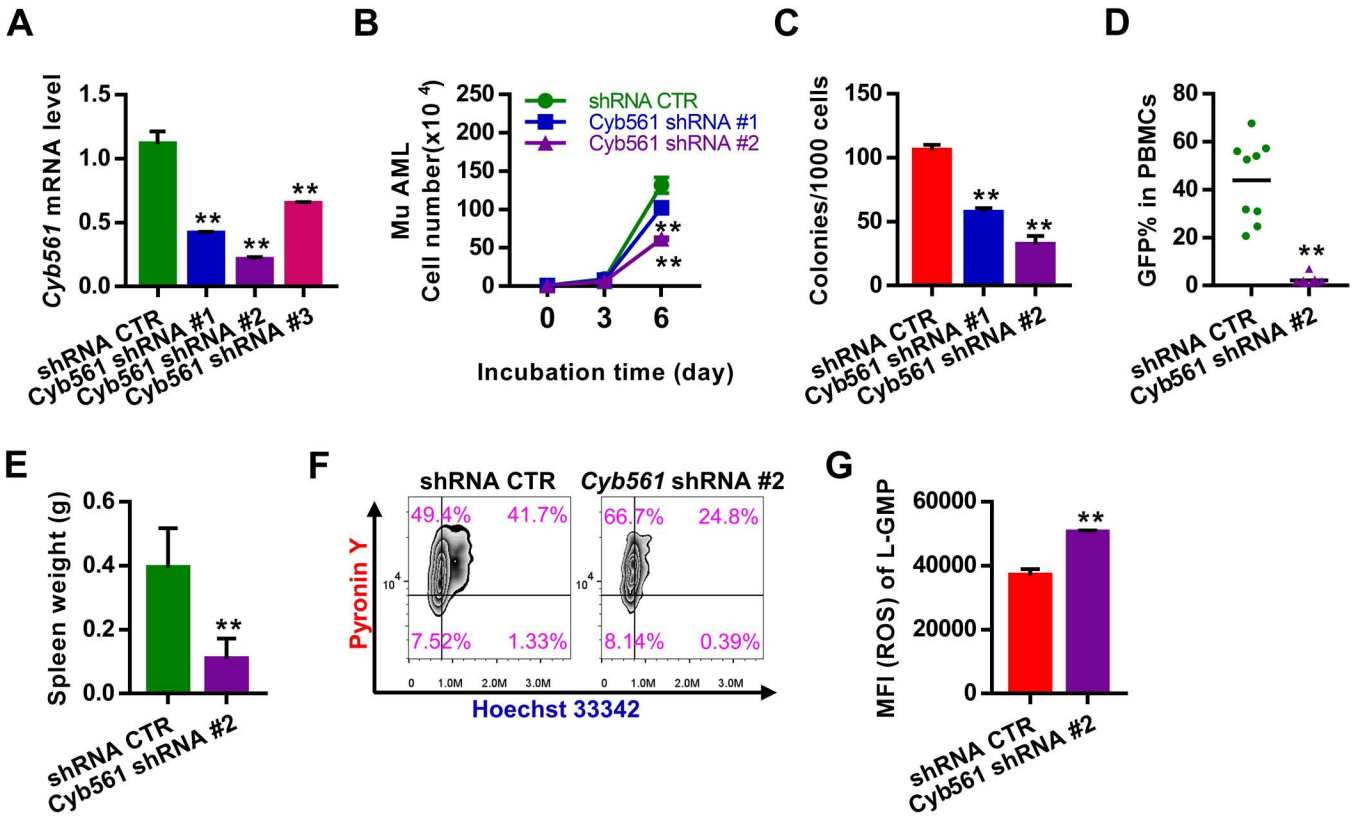
(C) Cell cycle phase distribution of LSCs in the BM from secondary recipients (n = 4).

(D) Representative flow cytometry analysis of cell cycle distribution of L-GMP in the BM from secondary recipients.

(E) Representative flow cytometry analysis of intracellular ROS in L-GMP from BM.

(F) The levels of ROS in L-GMP were evaluated by flow cytometry. MFI, mean fluorescence intensity (n = 5).

Data are representative of two or three independent experiments. All data are represented as mean ± SD. Two-tailed Student's *t*-tests were used to assess statistical significance ($*P < 0.05$; $** P < 0.01$; N.S., no significance).



Supplementary Figure 8. The effects of *Cyb561* on KMT2A::MLLT3 derived AML development. KMT2A::MLLT3-driven AML cells were transduced with inducible lentiviral particles containing shRNA control and *Cyb561* shRNAs. Transduced AML cells were treated with 1 μg/ml puromycin for 3 days for further experiments.

(A) qPCR analysis of *Cyb561* expression in AML cells. Results are normalized to *Gadph* expression and expressed relative to *Cyb561* expression in shRNA CTR group (n = 3).

(B) Knockdown of *Cyb561* suppresses the growth of mouse KMT2A:: MLLT3 AML cells (n = 3).

(C) Colony-forming assay of AML cells by knockdown of *Cyb561* (n = 3).

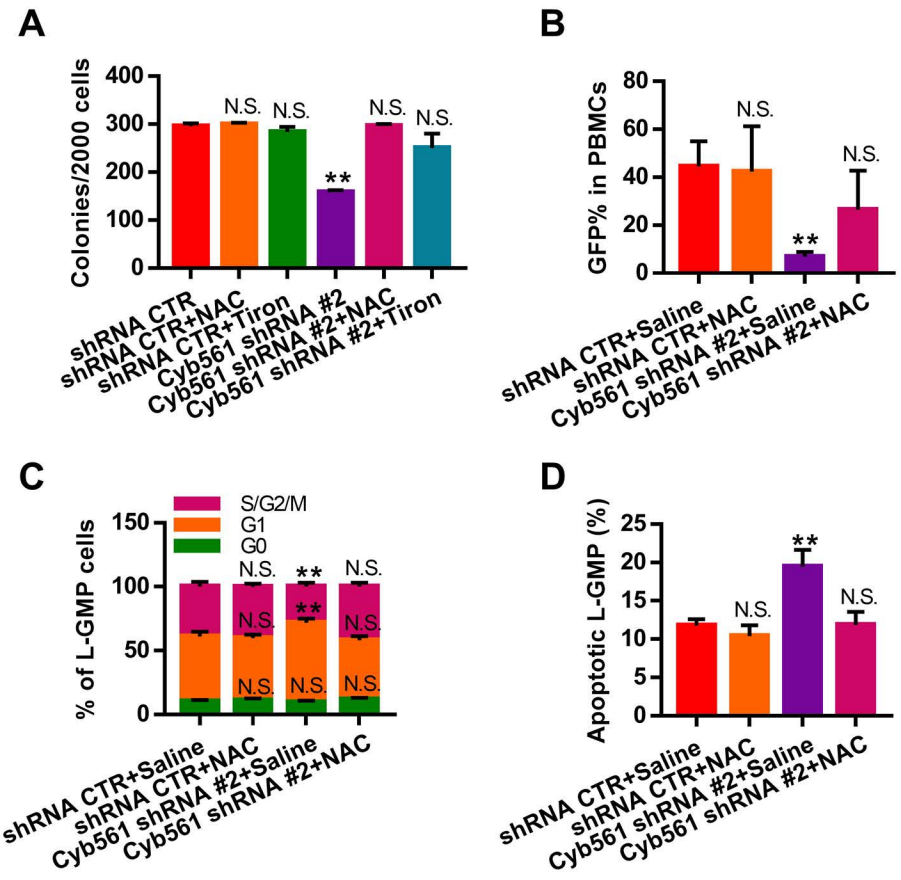
(D) GFP percentage in peripheral blood at week 3 after primary BMT with $1X10^5$ AML cells (n = 9).

(E) Spleen weight from primary recipients of shRNA CTR- or *Cyb561* shRNA #2-transduced AML cells (n = 5).

(F) Representative flow cytometry analysis of cell cycle distribution of L-GMP in the BM.

(G) Flow analysis of intracellular ROS levels in L-GMP cells from recipients (n = 4).

Data are representative of two or three independent experiments. All data are represented as mean ± SD. Two-tailed Student's *t*-tests were used to assess statistical significance ($*P < 0.05$; $** P < 0.01$; N.S., no significance).



Supplementary Figure 9. ROS scavenger impairs the functions of *Cyb561* on KMT2A::MLLT3-deriven L-GMPs.

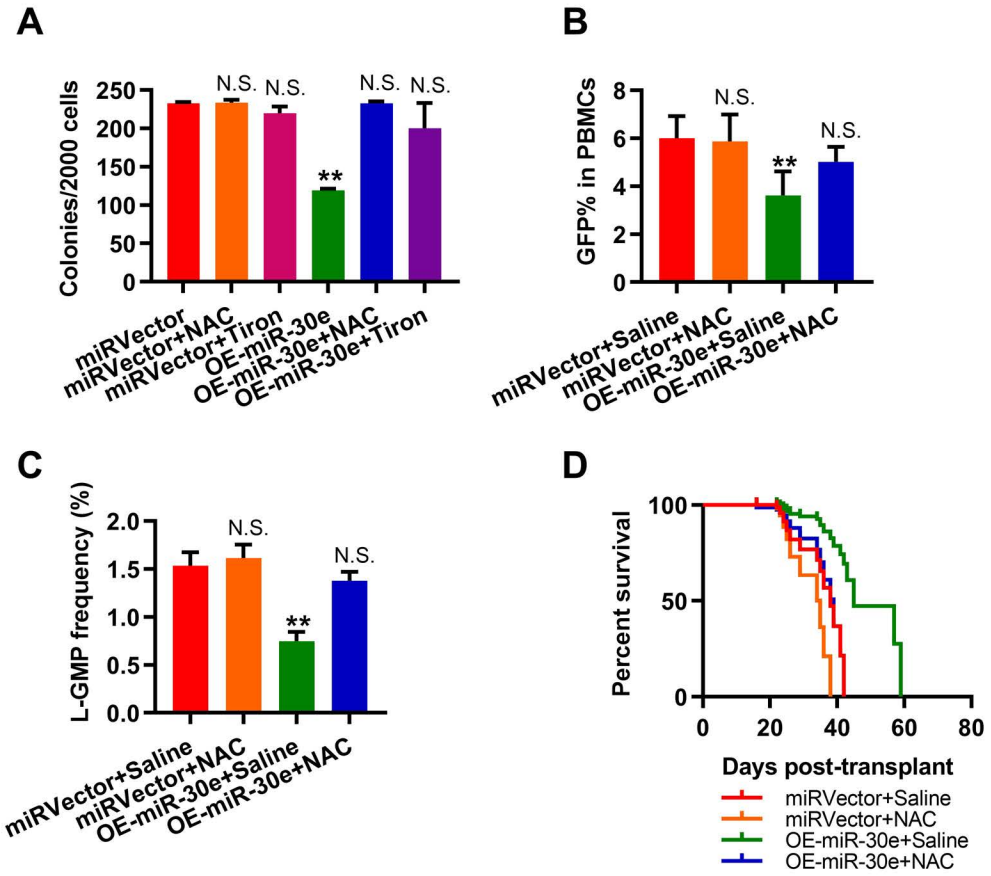
(A) Colony-forming assay of shRNA CTR- or *Cyb561* shRNA #2-transduced AML cells following treatment with NAC (1 mM) or Tiron (0.05 mM) (n = 3).

(B) GFP percentage in peripheral blood from recipients receiving $5X10^4$ AML cells infected with shRNA CTR and *Cyb561* shRNA #2 following treatment with saline or NAC (n = 5).

(C) Cell cycle phase distribution of L-GMP cells in BM from recipients with shRNA CTR or *Cyb561* shRNA #2 AML cells following treatment with saline or NAC (n = 5).

(D) Percentage of apoptotic L-GMP cells in the BM from primary recipients transplanted with shRNA CTR or *Cyb561* shRNA #2 AML cells following treatment with saline or NAC (n = 5).

Data are representative of two to three independent experiments. All data are represented as mean ± SD. Two-tailed Student's t-tests were used to assess statistical significance (** $P <$ 0.01; N.S., no significance).



Supplementary Figure 10. ROS scavenger impairs the functions of miR-30e on KMT2A::MLLT3-deriven L-GMPs.

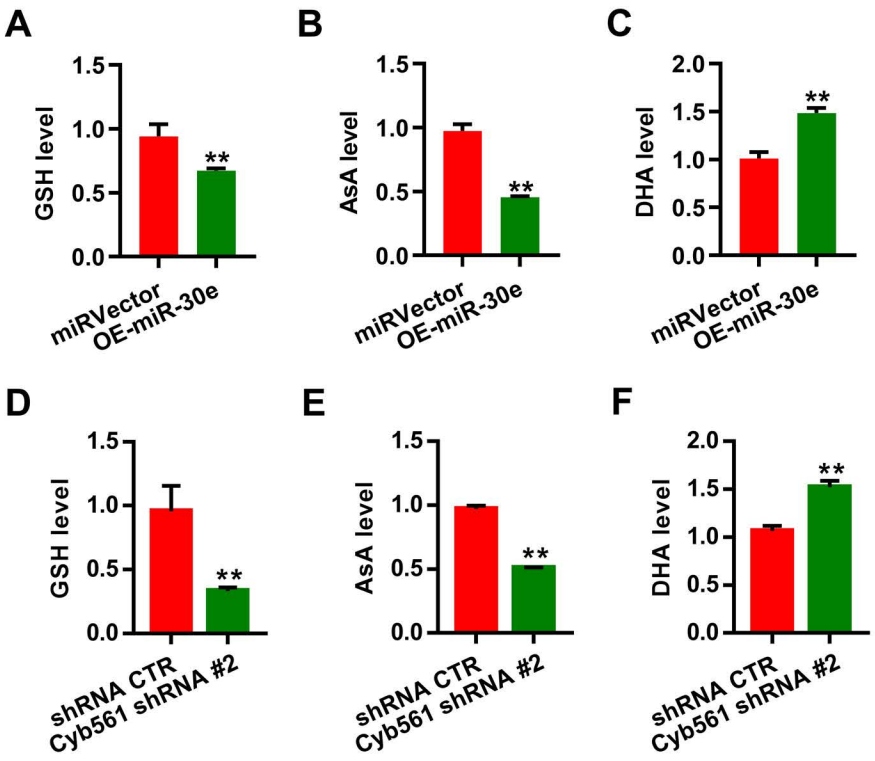
(A) Colony-forming assay of miRVector- or miR-30e-overexpressed AML cells treated with NAC (1 mM) or Tiron (0.05 mM) (n = 3).

(B) GFP percentage in peripheral blood at week 2 after transplantation with $1X10^5$ AML cells (n = 6).

(C) Frequency of L-GMP in the BM from miRVector- or miR-30e-overexpressed AML recipients with saline or NAC treatment (n = 5).

(D) Survival analysis of miRVector- or miR-30e-overexpressed AML recipient mice with saline or NAC treatment. Media survival was 38, 35, 45, and 39 days post-transplant for miRVector- or miR-30e-overexpressed AML recipients treated with saline or NAC, respectively ($P < 0.01$, Mantel-Cox test; n = 6).

Data are representative of two to three independent experiments. All data are represented as mean ± SD. Two-tailed Student's t-tests were used to assess statistical significance (** $P < 0.01$; N.S., no significance).

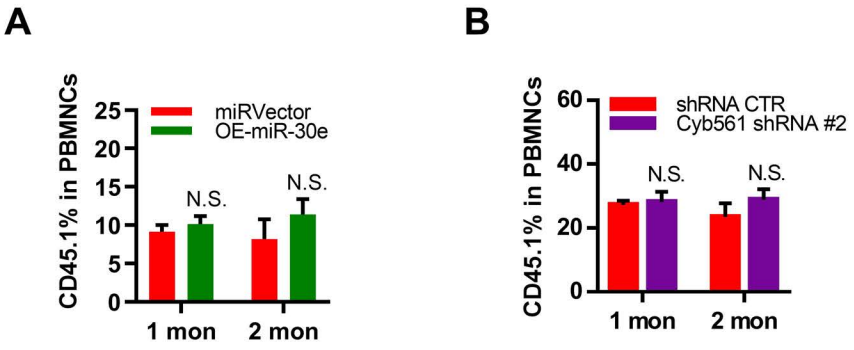


Supplementary Figure 11. Effects of miR-30e and *Cyb561* on the levels of GSH, AsA and DHA in mouse AML cells.

(A-C) The levels of GSH (A), AsA (B) and DHA (C) in mouse AML cells transduced with miRVector- or miR-30e. Results are expressed relative to the levels of GSH, AsA and DHA in the miRVector group (n = 3-4).

(D-F) The levels of GSH (D), AsA (E) and DHA (F) in mouse AML cells transduced with shRNA CTR- or *Cyb561* shRNA #2. Results are expressed relative to the levels of GSH, AsA and DHA in the shRNA CTR group (n = 3-4).

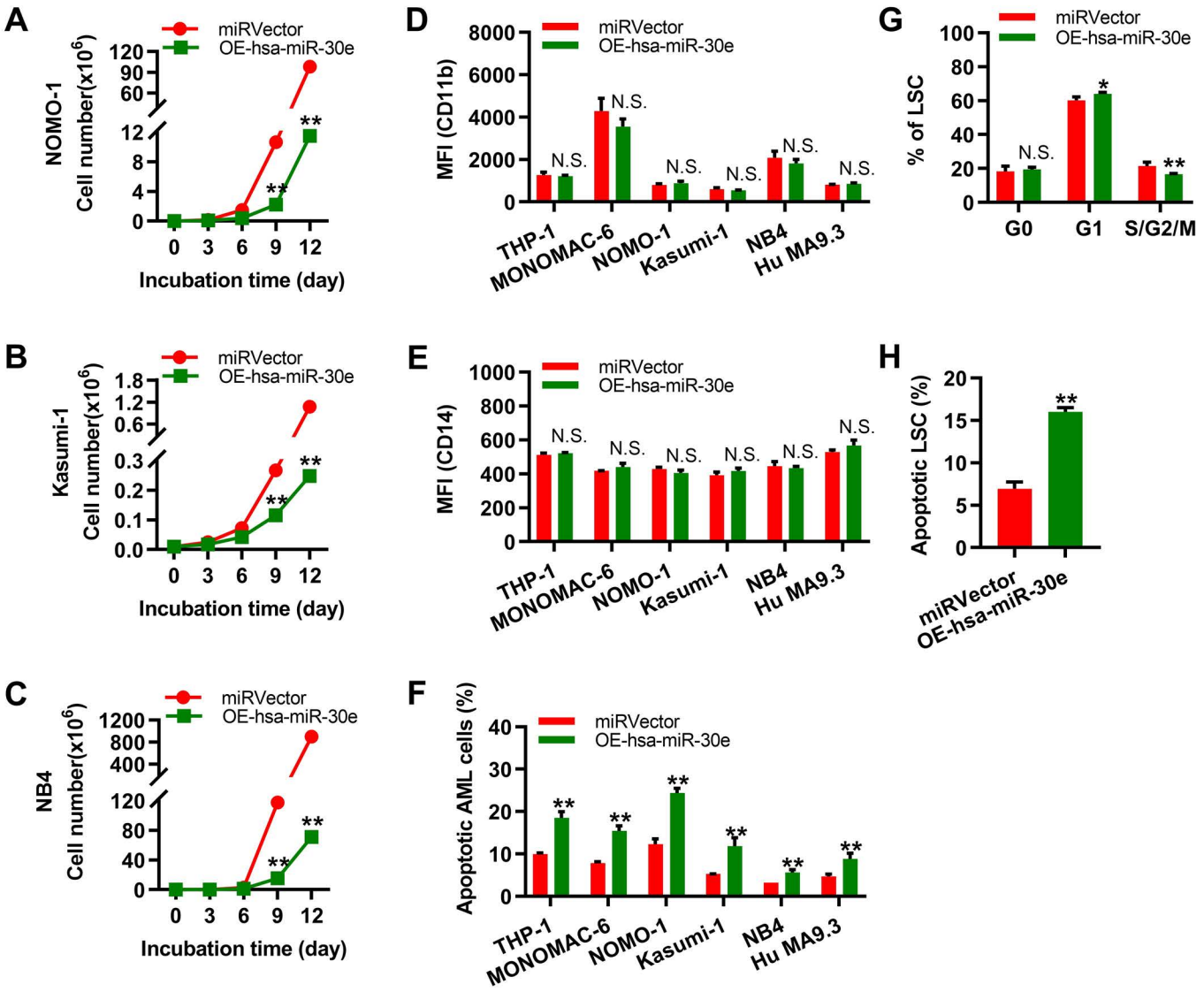
Data are representative of two or three independent experiments. All data are represented as mean ± SD. Two-tailed Student's *t*-tests were used to assess statistical significance (** $P < 0.01$).



Supplementary Figure 12. The effects of miR-30e and *Cyb561* on the repopulation capacity of HSCs.

(A) The effect of miR-30e on the repopulation capacity of HSCs. HSPCs were isolated from CD45.1 mouse injected with a single dose of 5-FU (150 mg/kg) and transduced with retroviruses containing miRVector or miR-30e. Transduced HSPCs were treated with 1 μg/ml puromycin for 3 days and transplanted into lethally-irradiated CD45.2 recipients. After 1 and 2 months of BMT, PBMNCs were isolated from the recipients and analyzed the ratio of CD45.1 by flow cytometry (n = 4).

(B) The role of *Cyb561* in the repopulation capacity of HSCs. HSPCs were isolated from CD45.1 mouse administrated with 5-FU (150 mg/kg) and transduced with inducible lentiviral particles containing shRNA CTR and *Cyb561* shRNA #2. Transduced HSPCs were treated with 1 μg/ml puromycin for 3 days and transplanted into lethally-irradiated CD45.2 recipients fed with drinking water containing 1 mg/ml doxycycline. After 1 and 2 months of BMT, PBMNCs were isolated from the recipients and analyzed the ratio of CD45.1 by flow cytometry (n = 3-4).



Supplementary Figure 13. The effects of forced-expression of miR-30e on human AML cells.

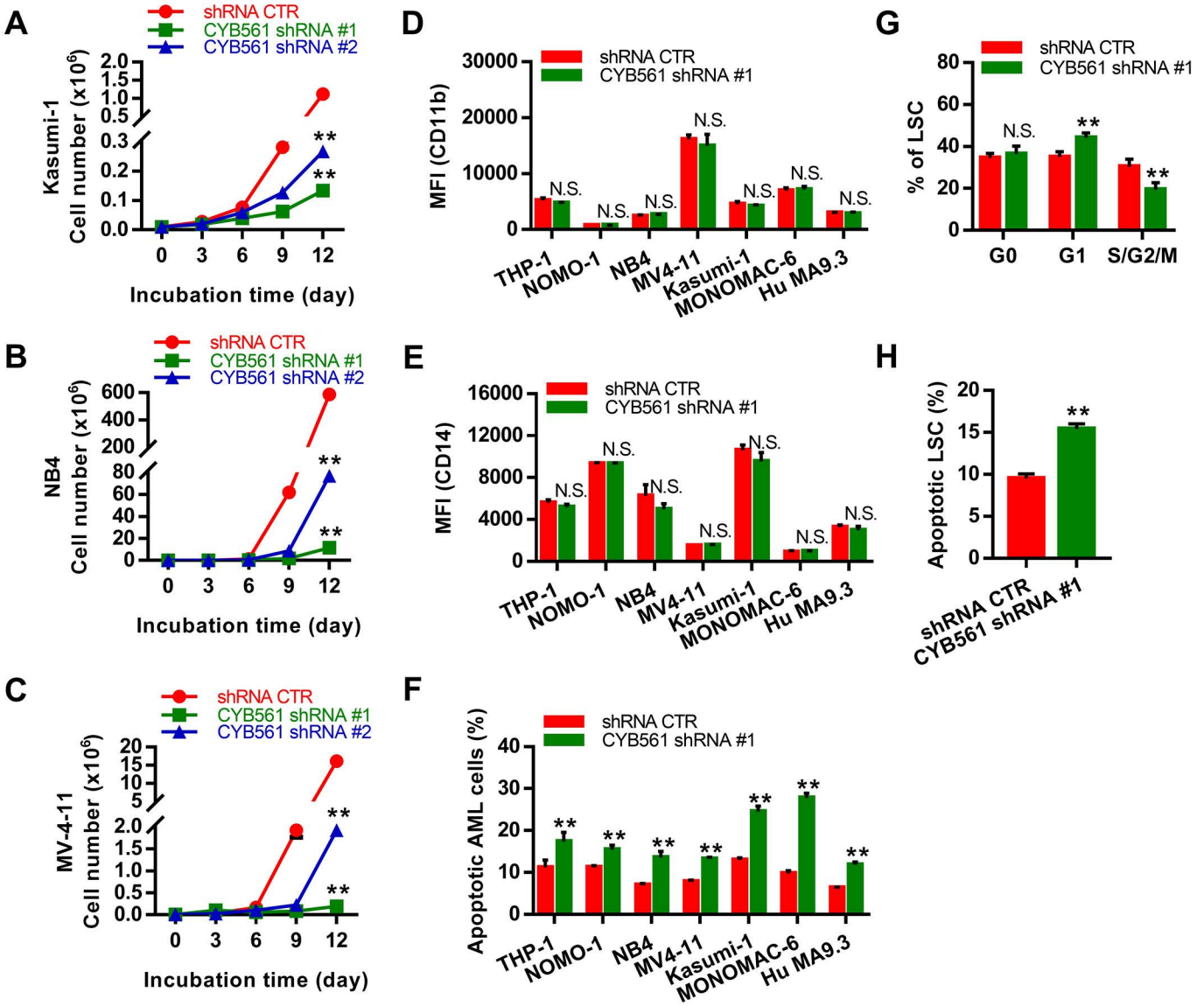
(A-C) Effects of overexpression of miR-30e on the growths of NOMO-1 (A), Kasumi-1 (B), and NB4 (C) cells (n = 3).

(D-E) Effects of overexpression of miR-30e on the differentiation of human AML cells. The transduced human AML cells were stained with CD11b (D) or CD14 (E) antibody and then performed flow cytometry analysis. MFI, mean fluorescence intensity (n = 4).

(F) Percentage of apoptotic AML cells transduced with miRVector or miR-30e (n = 4).

(G-H) Effects of miR-30e on the cell cycle (G) and apoptosis (H) of human LSCs ($CD34^+CD38^-$). Hu MA9.3 cells were transduced with miRVector or miR-30e, and then seeded into methylcellulose gel for colony formation. After 10 days, the colonies were isolated and analyzed by flow cytometry (n = 4).

Data are representative of two to three independent experiments. All data are represented as mean ± SD. Two-tailed Student's t-tests were used to assess statistical significance (* $P < 0.05$; ** $P < 0.01$; N.S., no significance).



Supplementary Figure 14. The effects of *CYB561* deficiency on human AML cells.

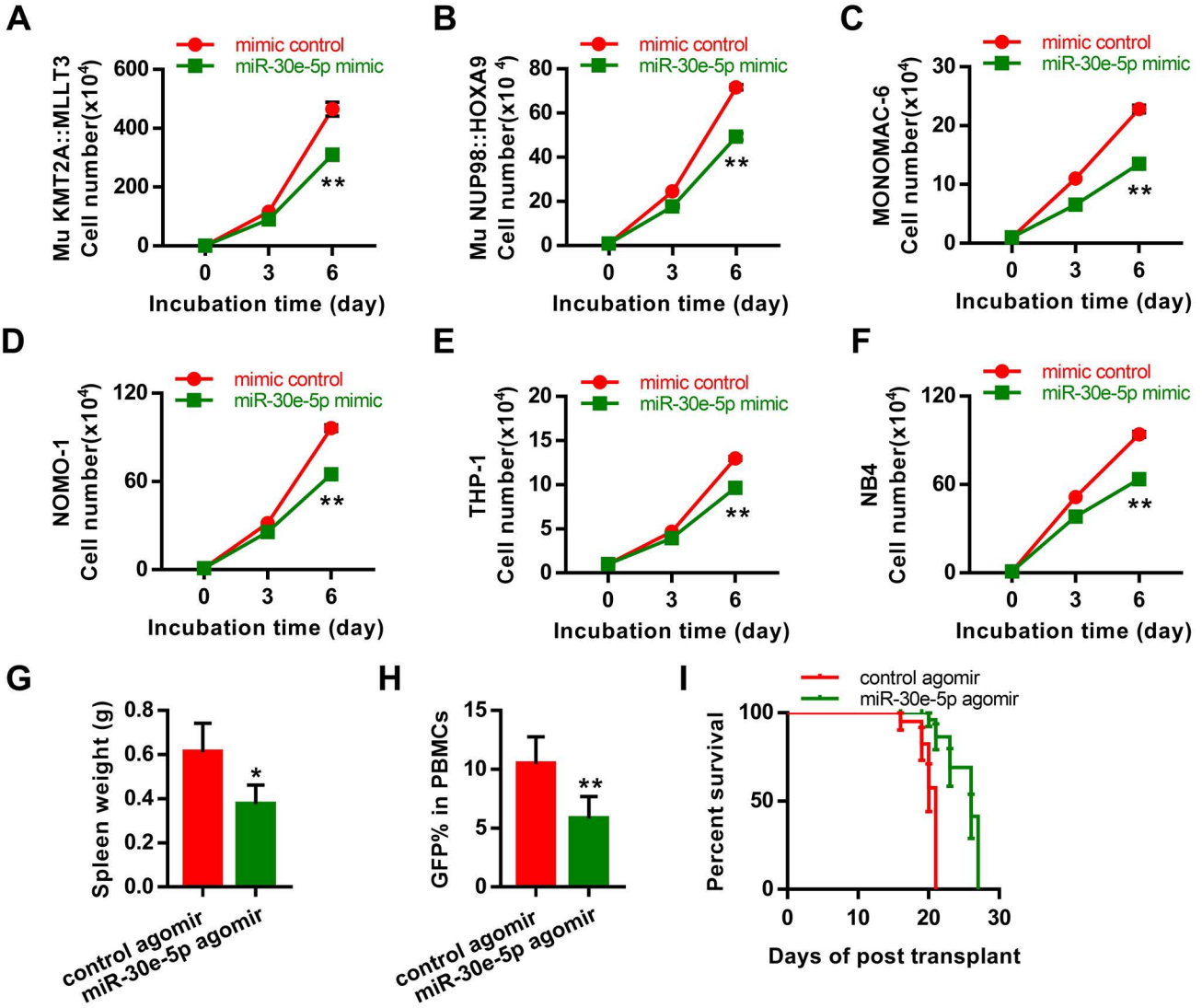
(A-C) Effects of *CYB561* knockdown on the growths of Kasumi-1 (A), NB4 (B), and MV-4-11 (C) cells (n = 3).

(D-E) Effects of *CYB561* knockdown on the differentiation of human AML cells. The transduced human AML cells were stained with CD11b (D) or CD14 (E) antibody and then performed flow cytometry analysis. MFI, mean fluorescence intensity (n = 3-4).

(F) Percentage of apoptotic AML cells transduced with shRNA CTR or CYB561 shRNA #1 (n = 3-4).

(G-H) Effects of *CYB561* knockdown on the cell cycle (G) and apoptosis (H) of human LSCs ($CD34^+CD38^-$). Hu MA9.3 cells were transduced with shRNA CTR or *CYB561* shRNA #1 and then seeded into methylcellulose gel for colony formation. After 10 days, the colonies were isolated and analyzed by flow cytometry (n = 4).

Data are representative of two to three independent experiments. All data are represented as mean ± SD. Two-tailed Student's t-tests were used to assess statistical significance (* $P < 0.05$; ** $P < 0.01$; N.S., no significance).

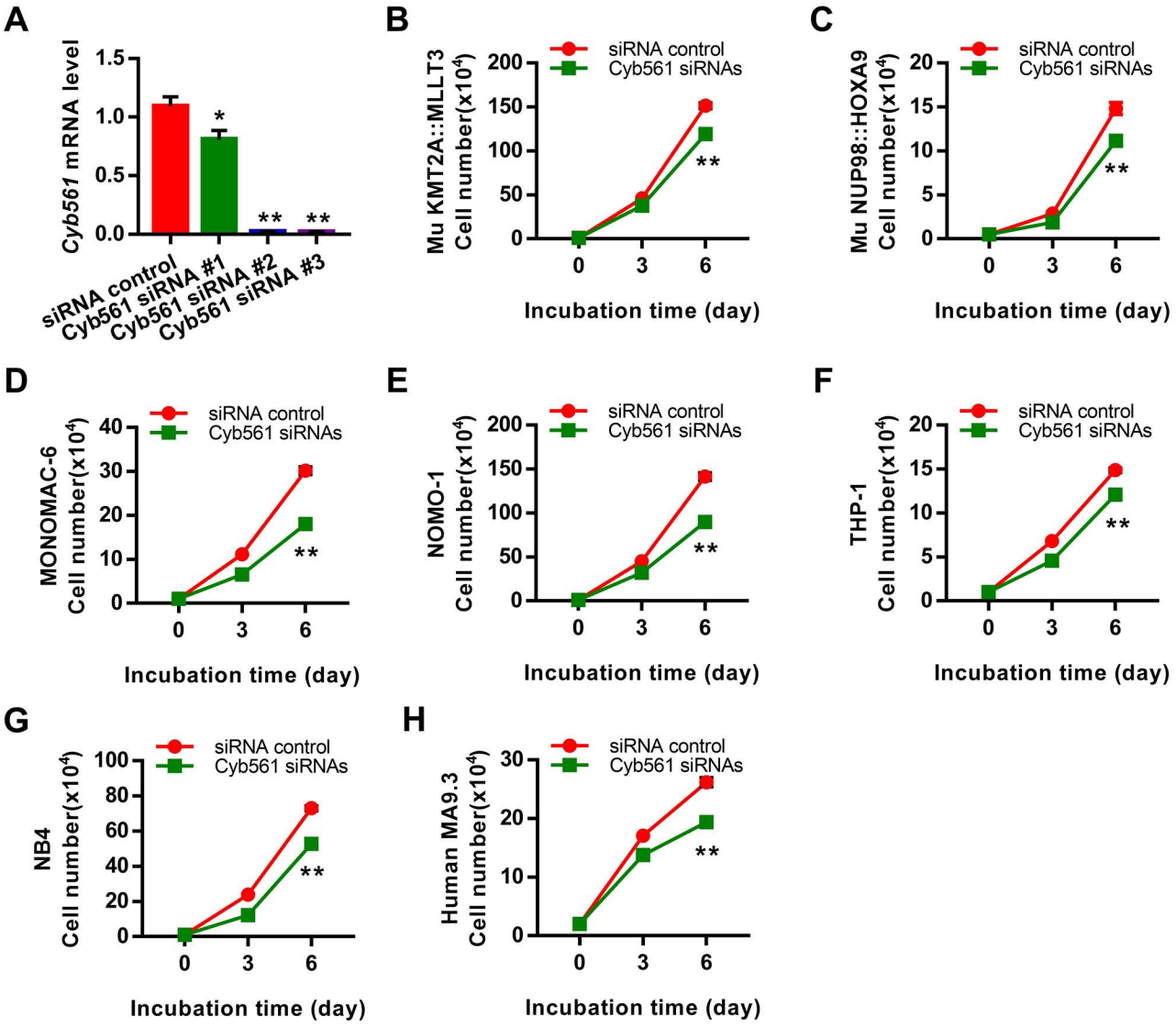


Supplementary Figure 15. Pharmacological targeting of miR-30e-5p inhibits mouse and human AML cell growth.

(A-F) Effects of miR-30e-5p mimics on the growths of mouse KMT2A::MLLT3 (A), mouse NUP98::HOXA9 (B), MOMONAC-6 (C), NOMO-1 (D), THP-1 (E), NB4 (F) cells (n = 3). The AML cells ($1x10^5$) were transfected with synthetic miRNA mimic control or miRNA-30e-5p mimic (100 nmol/L). After 24 hours of transfection, the transfected cell were performed the cell growth assay.

(G-I) Administration of miR-30e-5p mimic delays mouse KMT2A::MLLT3-derived AML development. The mouse KMT2A::MLLT3 AML cells ($1x10^5$) were transplanted into recipients. After 7 days of transplantation, the recipients were treated with 5 nmol miRNA control agomir or miR-30e-5p agomir twice a week continuously for 3 weeks. The spleen weight (G) (n = 5), GFP percentage in peripheral blood after 15 days of transplantation (H) (n = 6), survival time (I) (n = 5) were analyzed.

Data are representative of two to three independent experiments. All data are represented as mean ± SD. Two-tailed Student's t-tests were used to assess statistical significance (* $P < 0.05$; ** $P < 0.01$).



Supplementary Figure 16. Pharmacological targeting of CYB561 inhibits mouse and human AML cell growth. The AML cells were transfected with synthetic siRNA control or CYB561 siRNAs (100 nmol/L). After 24 hours of transfection, the transfected cell were performed the cell growth assay.

(A) qPCR analysis of *Cyb561* expression in moue KMT2A::MLLT3 AML cells transfected with siRNA control or Cyb561 siRNAs (n = 3).

(B-H) CYB561 siRNAs suppress the growths of mouse KMT2A::MLLT3 (B), mouse NUP98::HOXA9 (C), MONOMAC-6 (D), NOMO-1 (E), THP-1 (F), NB4 (G), Human MA9.3 (H) cells (n = 3).

Data are representative of two to three independent experiments. All data are represented as mean ± SD. Two-tailed Student's t-tests were used to assess statistical significance (* $P < 0.05$; ** $P < 0.01$).

Supplementary Table 1. List of primers for PCR.

Primer	Sequence (5' to 3')	Application
miR-30e-F	TCGACTCGAGACTTCTCAGAGAGAAAGGAAGTAACCC	PCR
miR-30e-R	TCGACTCGAGACATTAATTCTGACCCTGCATGAGG	PCR
hsa-miR-30e-F	TCGACTCGAGATTTGTAATTCTGACCCTGCATGAGG	PCR
hsa-miR-30e-R	TCGACTCGAGGACTTCTGAGAGAGGAGGGAAGGAC	PCR
mDock7-qF	CAACTGTAAACCAGGGACCTCTG	qPCR
mDock7-qR	GTAAGGCATCCTCACACCTTTTAGTA	qPCR
mSix1-qF	GGCCAAGGAAAGGGAGAACACCG	qPCR
mSix1-qR	ACTTTGGGGGGGTGAGAACTCCTCT	qPCR
mCyb561-qF	CTGCTGTTTAAATTGGGGTCCAAGTACA	qPCR
mCyb561-qR	AGTCAGCCTGAGCCAAGATGTAGA	qPCR
mCyb561-qF	TCAAGGAGATGCTCTGCTGGTGTAC	qPCR
mCyb561-qR	CGAACACAGCCACCAACCCCACCA	qPCR
mSix4-qF	GCAGCTTCACAAGGTAATCTTTCAGT	qPCR
mSix4-qR	CTGGCCTGAATTAGGAACGGTGTA	qPCR
mPrickle1-qF	CAACATCACAGGGGCATCAGTGGATG	qPCR
mPrickle1-qR	TGCAGCATGGAAGAGTTCAGAGTTC	qPCR
mRragd-qF	GCTTTGAAAGAAAAGGGCTGATTGAC	qPCR
mRragd-qR	CCATTGGGAGTAGCTCCTGTCTTC	qPCR
mEif5a2-qF	TCAGGATGGTTACCTTTCCCTGCTG	qPCR
mEif5a2-qR	CATGACAGACACCTGCACATCTTCA	qPCR
mIkzf2-qF	AGAAGTTTGTGGGGGAAAAGCTTATG	qPCR
mIkzf2-qR	CCAAGGTAGGTGATTGCATTGTTGATG	qPCR
mDock7_3'UTR-F1	CTAGTCTAGAAATACACACTTGTTCTATTCATTTGAAA	PCR
mDock7_3'UTR-R1	CTAGTCTAGACAACAACTTTCAGAGGAAAGTTAACAAC	PCR
mSix1_3'UTR-F1	CTAGTCTAGAGTGGGGAGATATTGGGGCCTTGAAGGGA	PCR
mSix1_3'UTR-R1	CTAGTCTAGAGATAAGAAAGAAATATATTAATATTGACACTTATA	PCR
mHoxa11_3'UTR-F1	CTAGTCTAGAGGCTCCAGCCTACTGGAATTGGGA	PCR
mHoxa11_3'UTR-R1	CTAGTCTAGATAGGTTATGGTTTCTTTATTTACAGTCT	PCR
mCyb561_3'UTR-F1	CGTAGCTAGCTCGCCCTCCTTGTCCTCTTGGCTCT	PCR
mCyb561_3'UTR-R1	CGTAGCTAGCCAGCTGTAGACATTTATTGATTCCTCTG	PCR
mSix4_3'UTR-F1	CGTAGCTAGCGCCCGATTCACTCCACATCCCTCCTT	PCR
mSix4_3'UTR-R1	CGTAGCTAGCTTCCTAGGTAAGTACTTAGTTTTGTGCT	PCR
mPrickle1_3'UTR-F1	CTAGTCTAGACCAAGTAGACATGGAGAGTCTTTGTTTA	PCR
mPrickle1_3'UTR-R1	CTAGTCTAGAGGTTTATTAAGCCTTTATTAAACACCTC	PCR

mIkzf2_3'UTR-F1	CTAGTCTAGAGCCTTTTCATTCCAAAGGGGACCCCT	PCR
mIkzf2_3'UTR-R1	CTAGTCTAGATTAAAGTGCGGGACCTTCTCCTAAAGATG	PCR
mCyb561_3'UTRBSm-F2	TGTACAGTCAAGCACGATGCTCTGGGCGACT	PCR
mCyb561_3'UTRBSm-R2	AGTCGCCCAGAGCATCGTGCTTGACTGTACA	PCR
mCyb561-F1	CTGACGGATCCATGGAGCACAGTTCTGCGTCTGTC	PCR
mCyb561-R1	CTGATGAATTCTCACTGGGGACTGGGGCTGTCTCCCTC	PCR
mCyb561 shRNA1-S1	CCGGGTCATAGGCATGATCTTCCTTCTCGAGAAGGAAGATCATGCCTATGACTTTTTG	PCR
mCyb561 shRNA1-AS1	AATTCAAAAAGTCATAGGCATGATCTTCCTTCTCGAGAAGGAAGATCATGCCTATGAC	PCR
mCyb561 shRNA2-S2	CCGGGCCAGACTACACAGAGAATTACTCGAGTAATTCTCTGTGTAGTCTGGCTTTTTG	PCR
mCyb561 shRNA2-AS2	AATTCAAAAAGCCAGACTACACAGAGAATTACTCGAGTAATTCTCTGTGTAGTCTGGC	PCR
hCYB561 shRNA1-S1	CCGGGAGTCCCTCCAGCCTGAATAACTCGAGTTATTCAGGCTGGAGGGACTCTTTTTG	PCR
hCYB561 shRNA1-AS1	AATTCAAAAAGAGTCCCTCCAGCCTGAATAACTCGAGTTATTCAGGCTGGAGGGACTC	PCR
hCYB561 shRNA4-S2	CCGGCGCCCACAGCACATCTTCTTTCTCGAGAAAGAAGATGTGCTGTGGGCGTTTTTG	PCR
hCYB561 shRNA4-AS2	AATTCAAAAACGCCCACAGCACATCTTCTTTCTCGAGAAAGAAGATGTGCTGTGGGCG	PCR
hHPRT-qF	CTCTCAACTTTAACTGGAAAGAATGTC	qPCR
hHPRT-qR	GAATTTCAAATCCAACAAAGTCTGGC	qPCR
mHPRT-qF	CTCATGGACTGATTATGGACAGGAC	qPCR
mHPRT-qR	GCAGGTCAGCAAAGAACTTATAGCC	qPCR

Supplementary Table 2. The characteristics of primary AML patients.

Sample ID	Gender	Age	FAB Subtypes
1	F	76	AML-M5
2	F	52	AML-M3
3	F	13	AML-M5
4	F	8	AML-M3
5	M	12	AML-M2
6	F	74	AML-M2
7	M	80	AML-M2
8	M	35	AML-M4
9	M	31	AML-M2
10	M	64	AML-M2
11	M	34	AML-M2
12	F	41	AML-M2
13	M	1	AML-M5
14	M	49	AML-M5
15	F	51	AML-M2
16	M	14	AML-M5
17	M	25	AML-M4
18	M	20	AML-M2
19	M	26	AML-M3
20	F	32	AML-M4
21	F	37	AML-M2
22	M	46	AML-M2
23	M	56	AML-M4
24	F	52	AML-M2
25	M	67	AML
26	F	58	AML-M2
27	M	51	AML
28	F	5	AML-M1
29	M	67	AML

Supplementary Table 3. Limiting diluting analysis of miRVector- and miR-30e-overexpressed LSCs from secondary recipients. Using the number of recipients that developed leukemia and total number of recipients transplanted at each cell dose, the LSC frequency was analyzed by ELDA online software.

Number of AML cells	miRVector	OE-miR-30e
5000	5/5	5/5
500	5/5	2/5
50	4/5	2/5
LSC frequency	1/31.1	1/529.9
95% confidence interval	1/92.3-10.5	1/1553.2-180.8

Supplementary Table 4. The differentially expressed genes in miRVector- and miR-30e-overexpressed LSCs.

Supplementary Table 5. The downregulated gene sets in miR-30e-overexpressed LSCs analyzed by GSEA.

Selected gene sets	Gene list of core enrichment
Electron transport chain	Atp5j, Cox8a, Atp5h, Atp5b, Atp5g3, Uqcrc2, Ndufs4,Ndufc2, mt-Atp6, Uqcrc1, Slc25a4, Atp5o, Ndufv1, mt-Co3, Atp5d, mt-Co2, Ndufa6, Cox4i1, Ndufs8, Uqcrfs1, Ndufa10, Ndufa9, Ndufb10, Ndufb2, Ndufb7, Cox17, Nudfa2, Cox5a, Slc25a5, Atp5l, Cox6a1, Ndufc1, Cox5b, Ndufa8, Uqcrb, Ndufb5, mt-Nd1, Cox7b, Cox7a2, mt-Atp8, Atp5e, mt-Nd3, Ndufb9, Cox6c, Uqcrq, Uqcr11, Ndufb6, Ndufs5, Uqcr10, Atp5g2, Ndufa5, Ndufv3, Ndufa12, Ndufa1, Ucp2, Ucp3, Ndufs6, Atpif1, Ndufb4,Cox7a1, Slc25a27
Oxidative phosphorylation	Atp5j, Atp5h, Atp5b, Atp5g3, Ndufs4, Ndufc2, mt-Atp6, Atp5o, Ndufv1, Atp5d, Atp5g1, Ndufa6, Ndufs8, Ndufa10, Ndufa9, Ndufb10, Ndufb2, Ndufb7, Ndufa2, Ndufb8, tp5l, Ndufc1, Ndufa8, Ndufb5, mt-Nd1, mt-Atp8, Atp5e, mt-Nd3, Ndufb9, Ndufb6, Ndufs5, Atp5g2, Ndufa5, Ndufv3, Ndufa11, Ndufs6
Glutathione metabolism	Gclm, Ggt5, Oplah, Gstt2, Anpep, Gstm2, Gpx3, Ggt1, Gpx2
Cytoplasmic ribosomal proteins	Rpl22, Rpl19, Rps16, Rpl26, Tps27, Rps24, Rpl30, Rps27a, Fau, Rpl32, Rpl13a, Rps17, Rps17, Rps6, Rps15, Rpl7a, Rpl35, Rps20, Rpl13, Rpl37, Rpl24, Rplp1, Rps28, Rpl17, Rps10, Rpl23a, Rpl36, Rpl27, Rpl28, Rpl12, Rps15a, Rpl39, Rpl37a, Rpl11, Rpl35a, Rpl36a, Rpl31, Rpl38, Rps7, Rpl41, Rps29
Cholesterol biosynthesis	Nsdhl, Hmgcs1, Fdps, Mvk, Fdft1, Mvd, Sc5d, Lss, Pmvk, Idi1, Cyp51, Msmo1, Dhcr7, Sqle

Supplementary Table 6. KEGG pathway enrichment of differentially expressed genes ranked by q-value. Rich factor represents the ratio of the enriched differentially expressed genes' number in this pathway to the number of genes annotated in this pathway.

Pathway	q-value	p value	Rich factor
Malaria	7.55E-05	2.59E-07	5.057815938
Fluid shear stress and atherosclerosis	0.000470694	3.22E-06	2.92946786
Transcriptional misregulation in cancer	0.000502776	5.17E-06	2.63265804
Type I diabetes mellitus	0.000569046	7.80E-06	3.872390328
Graft-versus-host disease	0.000733392	1.26E-05	3.920521733
Allograft rejection	0.0013238	3.63E-05	3.768220763
Kaposi's sarcoma-associated herpesvirus infection	0.00134523	3.22E-05	2.349127782
African trypanosomiasis	0.001502497	3.09E-05	4.720628209
Hematopoietic cell lineage	0.001718836	5.30E-05	3.053014983
Cell adhesion molecules (CAMs)	0.005511213	0.000226488	2.345744264
Antigen processing and presentation	0.005812762	0.000218974	2.915682129
Cellular senescence	0.005812827	0.000199069	2.282071648
Apoptosis	0.006944498	0.000332955	2.429735108
Herpes simplex infection	0.007206583	0.000320841	2.144708489
Autoimmune thyroid disease	0.007451151	0.000408282	2.983174771
AGE-RAGE signaling	0.007644706	0.000445069	2.643551797
Osteoclast differentiation	0.007805704	0.000400978	2.452513874
Phagosome	0.009342159	0.000575886	2.196592895
Cholinergic synapse	0.00983558	0.000639986	2.485640517
MAPK signaling	0.014260693	0.00097676	1.867234788
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	0.015456932	0.001164563	2.792484293
Toll-like receptor signaling	0.01573864	0.001131888	2.503363444
Viral myocarditis	0.016247188	0.001279744	2.651711
Dopaminergic synapse	0.017994567	0.001479005	2.2530271
Focal adhesion	0.018422443	0.001640354	2.002690755
Hypertrophic cardiomyopathy (HCM)	0.018799075	0.00160951	2.587814259
Human papillomavirus infection	0.020861571	0.002000425	1.744279045
Viral carcinogenesis	0.020872274	0.001929971	1.926355009
Rap1 signaling	0.021170819	0.002102581	1.939225203
Steroid biosynthesis	0.022807979	0.002499505	4.347947035
TNF signaling	0.023503165	0.002495199	2.316196084
Dilated cardiomyopathy (DCM)	0.024201058	0.00248641	2.468834293
Acute myeloid leukemia	0.024294454	0.002828806	2.633973711
HTLV-I infection	0.02490633	0.002814756	1.795891166
Chagas disease (American trypanosomiasis)	0.030734971	0.003683986	2.290205765
Adrenergic signaling in cardiomyocytes	0.031560786	0.003891056	2.065274841
PI3K-Akt signaling	0.040129766	0.005359797	1.652219873
Leishmaniasis	0.041056229	0.005342934	2.541876728
Apelin signaling	0.041172191	0.005217024	2.050199843
cAMP signaling	0.047788523	0.006546373	1.84511864
Sphingolipid signaling	0.049526059	0.006954001	2.082630092

Supplementary Table 7. Top downregulated miR-30e-5p target genes identified from RNA-seq in LSCs overexpressed with miR-30e or miRVector. The binding sites of downregulated genes were analyzed by Targetscan (https://www.targetscan.org/vert_80/) and miRDB (https://mirdb.org/) online software.

Predicted target	Binding site of target's 3'UTR	Predicted consequential pairing of target region (5' to 3') (in red)	Log_2FC (miRVector vs OE-miR-30e)	Context+ + Score percentile
Hoxa11	1145-1152	UUUAAAGGCUCUAUCUGUUUACA	-5.41	87
Cyb561	1598-1605	ACUUGUACAGUCAAGUGUUUACA	-4.73	90
Dock7	392-399	UAUUAAAUAUGUGAAUGUUUACA	-3.44	92
Ikzf2	544-550	UUGUUGUGGGUUCUGUGUUUACC	-2.99	32
Prickle1	118-125	UCUGUGGCCCCACUGUGUUUACA	-2.78	85
Six4	410-416	GUUUGUUUGUUUGUUUGUUUACU	-2.45	84
Six1	555-561	UGAUGGGAAGAUUAAGUUUACAU	-1.94	85
	1248-1254	UUAGAAAUGCAGUUUUGUUUACC		77

Supplementary Table 8. Limiting diluting analysis of shRNA CTR- and *Cyb561* shRNA #2-transduced LSCs from secondary recipients. Using the number of recipients that developed leukemia and total number of recipients transplanted at each cell dose, the LSC frequency was analyzed by ELDA online software.

Number of AML cells	miRVector +pMIBSD	miRVector +pMIBSD-*Cyb561*	OE-miR-30e +pMIBSD	OE-miR-30e +pMIBSD-*Cyb561*
5000	6/6	6/6	6/6	6/6
500	6/6	6/6	3/6	5/6
50	5/6	4/6	2/6	5/6
LSC frequency	1/28.4	1/46.0	1/474.3	1/124.7
95% confidence interval	1/76.2-10.8	1/127.7-16.8	1/1235.3-182.3	1/343.3-45.5

Supplementary Table 9. Limiting diluting analysis of shRNA CTR- and *Cyb561* shRNA #2-transduced LSCs from secondary recipients. Using the number of recipients that developed leukemia and total number of recipients transplanted at each cell dose, the LSC frequency was analyzed by ELDA online software.

Number of AML cells	shRNA CTR	*Cyb561* shRNA #2
5000	6/6	6/6
500	6/6	4/6
50	5/6	2/6
LSC frequency	1/28.4	1/335.3
95% confidence interval	1/76.2-10.8	1/826.6-136.2

Supplementary Table 10. List of the sequences of miRNA agomirs and mimics.

Primer	Sequence (5’ to 3’)	Application
control agomir/sense	UUGUACUACACAAAAGUACUG	In vivo
control agomir/antisense	GUACUUUUGUGUAGUACAAUU	In vivo
miR-30e-5 agomir/sense	UGUAAACAUCCUUGACUGGAAG	In vivo
miR-30e-5p agomir/antisense	UCCAGUCAAGGAUGUUUACAUU	In vivo
miRNA negative control/sense	UUUGUACUACACAAAAGUACUG	In vitro
miRNA negative control /antisense	CAGUACUUUUGUGUAGUACAAA	In vitro
miR-30e-5p mimic/sense	UGUAAACAUCCUUGACUGGAAG	In vitro
miR-30e-5p mimic /antisense	CUUCCAGUCAAGGAUGUUUACA	In vitro

Supplementary Table 11. List of antibodies for flow cytometry.

Supplier	Antibody	Catalog Number
ThermoFisher	Mouse Hematopoietic Lineage Biotin Panel	88-7774-75
ThermoFisher	CD117 (c-Kit) Monoclonal Antibody (2B8), PE-Cyanine7	25-1171-82
ThermoFisher	CD34 Monoclonal Antibody (RAM34), eFluor 660	50-0341-82
Biolegend	APC/Fire™ 750 anti-mouse CD16/32	156621
Biolegend	PE Annexin V	640908
ThermoFisher	Streptavidin PerCP-Cyanine5.5	45-4317-82
ThermoFisher	CD11b Monoclonal Antibody (M1/70), eFluor™ 450	48-0112-80
ThermoFisher	CD14 Monoclonal Antibody (61D3), eFluor 450	48-0149-41
Biolegend	APC/Cy7 anti-human CD34 Antibody	343614
Biolegend	PE/Cy7 anti-human CD38 Antibody	356608
Biolegend	FITC anti-mouse CD45.1 Antibody	110705
Biolegend	Ly-6A/E (Sca-1) Monoclonal Antibody (D7), APC	17-5981-82
Biolegend	CD34 Monoclonal Antibody (RAM34), FITC	11-0341-85
Biolegend	CD135 (Flt3) Monoclonal Antibody (A2F10), PE	12-1351-82