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

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Abstract

Leukemia stem cells (LSC) represent a crucial and rare subset of cells present in acute myeloid leukemia (AML); they play a pivotal role in the initiation, maintenance, and relapse of this disease. Targeting LSC holds great promise for preventing AML relapse and improving long-term outcomes. However the precise molecular mechanisms governing LSC self-renewal are still poorly understood. Here, we present compelling evidence that the expression of miR-30e-5p, a potential tumor-suppressive microRNA, is significantly lower in AML samples than in healthy bone marrow samples. Forced expression of miR-30e effectively inhibits leukemogenesis, impairs LSC self-renewal, and delays leukemia progression. Mechanistically, *Cyb561* acts as a direct target of miR-30e-5p in LSC, and its deficiency restricts the self-renewal of LSC by activating reactive oxygen series signaling and markedly prolongs recipients' survival. Moreover, genetic or pharmacological overexpression of miR-30e-5p or knockdown of *Cyb561* suppresses the growth of human AML cells. In conclusion, our findings establish the crucial role of the miR-30e-5p/Cyb561/ROS axis in finely regulating LSC self-renewal, highlighting *Cyb561* as a potential therapeutic target for LSC-directed therapies.

Introduction

Acute myeloid leukemia (AML) is a highly aggressive and often fatal blood malignancy primarily caused by genetic mutations in hematopoietic stem cells (HSC) or committed progenitor compartments.[1,2] Despite ongoing efforts, the standard therapy for most types of AML has not shown significant improvements, with a 5-year overall survival rate of approximately 24% in the USA.[3] Leukemia stem cells (LSC), an indispensable subset of AML cells with a limitless capacity of self-renewal and differentiation block, play a key role in the initiation, maintenance, and propagation of AML.[4] Targeting LSC has emerged as a promising strategy to address AML relapse and enhance long-term treatment outcomes.

MicroRNA (miRNA, miR), typically consisting of 18-24 nucleotides, which function by binding to and cleaving mRNA or inhibiting their translation. Accumulating evidence supports the critical role of miRNA in regulating LSC self-renewal and differentiation.[5-7] Among them, miR-30e-5p is implicated in many important biological regulation processes, such as cancer development and metastasis, epithelial-mesenchymal transition, fatty acid metabolism, and osteogenesis.[8-12] Previous studies have documented lower levels of expression of miR-30e-5p in patients with chronic myeloid leukemia. Additionally, enforced expression of miR-30e-5p has demonstrated inhibitory effects on proliferation and induction of apoptosis in K562 cells.[13] Another study highlighted the involvement of circPVT1, which regulates the miR-30e/DLL4 pathway, in suppressing the proliferation of T-cell acute lymphoblastic leukemia cells.[14] However, the precise roles of miR-30e-5p in LSC initiation and maintenance and its therapeutic potential in AML remain largely unexplored.

In this study, we aimed to investigate the roles of miR-30e-5p in LSC initiation and maintenance in *KMT2A::MLLT3*-induced leukemia, as well as explore the potential of this microRNA as a therapeutic target in human AML cells. We

demonstrated that miR-30e-5p is expressed at lower levels in human AML bone marrow (BM) samples compared to healthy samples. Forced expression of miR-30e delays the onset of *KMT2A::MLLT3*-driven leukemia by reducing cycling LSC and promoting LSC apoptosis. Using RNA-sequencing analysis, we found that endogenous *Cyb561* is significantly suppressed in the LSC overexpressing miR-30e compared with LSC expressing empty retroviral miRVector. By quantitative PCR (qPCR), reporter assays, and functional assays, we identified *Cyb561* as a direct downstream target of miR-30e-5p in leukemogenesis. Consistently, knockdown of *Cyb561* resulted in reduced colony formation capacity *in vitro*, decreased LSC frequency, and delayed progression of mouse AML *in vivo*.

Moreover, the miR-30e-5p/Cyb561 signaling pathway was found to enhance intracellular reactive oxygen species (ROS) levels, while concurrently decreasing glutathione and ascorbate levels. The ROS scavenger N-acetyl-L-cysteine (NAC) impeded the roles of miR-30e-5p and *Cyb561* in leukemia progression by regulation of cell cycle and apoptosis of LSC. In addition, both genetic and pharmacological upregulation of miR-30e-5p hindered the growth of human AML cells. Consistent with these findings, we observed a significant upregulation of CYB561 expression in AML patients, and high-level expression of CYB561 correlated with shorter overall survival in AML patients. Inhibition of CYB561 had suppressive effects on human AML cell growth *in vitro*. Together, our study provides compelling evidence supporting the essential role of the miR-30e-5p/Cyb561/ROS signaling pathway in the initiation and maintenance of LSC in *KMT2A::MLLT3*-induced leukemia. Furthermore, we highlight the potential of Cyb561 as a therapeutic target for LSC in AML.

Methods

Mice

C57BL/6J mice were purchased from VITALSTAR (Beijing, China). All the animal studies conducted in this research were approved by the Animal Care and Use Committee at Shanghai University.

Cell cultures

293T cells were cultured in DMEM (high glucose) containing 10% fetal bovine serum. THP-1, MONOMAC-6, NOMO-1, MV4-11, and NB-4 cells were cultured in RPMI1640 plus 10% fetal bovine serum. Human MA9.3 leukemic cells were cultured in IMDM plus 20% fetal bovine serum with defined cytokines.[15] Enriched fresh hematopoietic stem and progenitor cells (HSPC) were cultured *in vitro* in StemSpan (STEMCELL Technologies, Vancouver, Canada) supplemented with 10 μg/mL heparin (Sigma, St Louis, MO, USA), 10 ng/mL murine stem cell factor, 20 ng/mL murine thrombopoietin, 20 ng/mL human insulin-like growth factor-II, 10 ng/mL murine fibroblast growth factor-1, and 100 ng/mL human angiopoietin-like protein 3. All recombinant proteins were purchased from PeproTech (Rock Hill, NJ, USA) or Genscript (Nanjing, China). All cell culture products were obtained from Jet Biofil (Guangzhou, China) unless otherwise specified in the text and figure legends.

Plasmids

KMT2A::MLLT3 (previously called *MLL-AF9*) was cloned into the retroviral vector pMIGR1 containing green fluorescent protein (GFP). The entire loci of mouse and human miR-30e, containing 100-bp upstream and downstream native flank sequences, were amplified by PCR from genomic DNA; then cloned into pMXs-miR-Puro (miRVector) retroviral vector. To generate short hairpin (sh)RNA-expressing plasmids targeting mouse and human CYB561, we cloned shRNA control and CYB561 shRNA into Age I and EcoR I sites of Tet-pLKO-puro vector (Addgene, USA). To construct the luciferase reporters for identifying true targets of miR-30e-5p, the sequences of target 3' untranslated regions (3'UTR) were amplified from genomic DNA of C57/B6J BM cells by PCR using specific primers and cloned into the pGL3-control vector (Promega). To generate the mutant of *Cyb561* 3'UTR, point mutations in the miR-30e-5p binding site were introduced by PCR and cloned into the pGL3-control vector. The reporter plasmids were validated by DNA sequencing. For the ectopic expression of *Cyb561*, the coding sequence of *Cyb561* was amplified from mouse BM cDNA and then cloned into a retroviral vector pMIBSD containing the selective antibiotic blasticidin. All the primers for plasmid cloning are listed in *Online Supplementary Table S1*.

Results

miR-30e-5p expression is downregulated in patients with acute myeloid leukemia

Previous clinical reports showed that miR-30e-5p is upregulated in patients with B-cell acute lymphoblastic leukemia and childhood acute lymphoblastic leukemia but downregulated in patients with chronic myeloid leukemia.[13,16,17] To assess the potential clinical relevance of miR-30e-5p expression levels in AML samples and healthy samples, we carried out qPCR analysis of the expression of mature miR-30e-5p and miR-30e-3p. Our findings revealed a significant downregulation of miR-30e-5p in AML BM samples compared to healthy BM samples (Figure 1A, *Online Supplementary Table S2*). No significant difference was observed in the expression of miR-30e-3p between AML samples and healthy samples (*Online Supplementary Figure S1A*). To assess the expression levels of miR-30e-5p in different subtypes of AML, defined according to the French-American-British (FAB) classification, and determine its prognostic value for overall survival, we searched clinical databases

online (*http://ualcan.path.uab.edu/index.html*) and found that the expression of miR-30e-5p was lower in human AML-M3, -M4, and -M5 subtypes than in AML-M2 (*Online Supplementary Figure S1B*). The overall survival of AML patients was not significantly related to the expression level of miR-30e-5p (*Online Supplementary Figure S1C*). To provide more conclusive evidence for the decreased expression of miR-30e-5p in LSC, we examined a mouse AML model driven by the *KMT2A::MLLT3* fusion oncogene. Our results demonstrated a reduction in miR-30e-5p expression in LSC-enriched granulocyte-monocyte progenitors (referred to as L-GMP, $Lin^-GFP^+c\text{-}Kit^+CD34^+CD16/32^+$) compared to HSPC and granulocyte-monocyte progenitors (GMP) (Figure 1B). Furthermore, we observed the downregulation of endogenous miR-30e-5p in HSPC expressing various oncogenes (e.g., *HOXA9*, *MESI1*, and *NUP98::HOXA9*) (Figure 1C). These findings in the AML mouse model are consistent with the results obtained from human AML samples, suggesting a potentially significant role for miR-30e-5p in AML.

Overexpression of miR-30e delays the development of *KMT2A::MLLT3*-driven leukemia

To determine the role of miR-30e in AML cells, we enriched $Lin^-Sca\text{-}1^+$ HSPC by a single intraperitoneal dose of 5-fluorouracil. These cells were subsequently co-transduced with retroviral particles containing *KMT2A::MLLT3*/miRVector and *KMT2A::MLLT3*/miR-30e. Overexpression of miR-30e in HSPC resulted in a 5.36-fold increase in miR-30e-5p expression compared to the level in miRVector-HSPC (*Online Supplementary Figure S2A*). Colony-forming/replating assays showed that forced expression of miR-30e suppressed *KMT2A::MLLT3*-induced immortalization of mouse HSPC (Figure 2A, *Online Supplementary Figure S2B*). To explore the roles of miR-30e in AML *in vivo*, equal numbers of *KMT2A::MLLT3*/miRVector- and *KMT2A::MLLT3*/miR-30e-transduced HSPC were transplanted into irradiated recipients. Both sets of HSPC developed AML in recipient mice with full penetrance; however, the onset was significantly delayed in miR-30e-overexpressing AML compared to miRVector-AML (median survival, 78 days *vs.* 52 days, respectively; $P<0.01$) (Figure 2B, C). The expression level of miR-30e-5p was elevated 3.6-fold in miR-30e-overexpressing AML cells (*Online Supplementary Figure S2C*). Consistent with these findings, other parameters of AML severity, such as peripheral blood white cell count and spleen weight, were also reduced (*Online Supplementary Figure S2D-F*). In line with the primary BM transplantation results, the secondary recipients of miRVector developed AML significantly faster than the miR-30e-overexpressing secondary recipients (median survival, 41 days *vs.* 53 days, respectively; $P<0.01$) (*Online Supplementary Figure S2G*).

The frequency of LSC is thought to be associated with patients' prognosis as well as leukemia progression in murine models. To further investigate the impact of miR-30e on LSC, we analyzed L-GMP (i.e., a LSC-enriched population) frequency in the secondary recipients overexpressing the miRVector or miR-30e. The results showed that forced expression of miR-30e resulted in a lower LSC frequency (Figure 2D, *Online Supplementary Figure S3*), indicating a potential role for miR-30e in modulating LSC self-renewal. Additionally, cell cycle analysis demonstrated a decreased proportion of miR-30e-overexpressing LSC in the S/G2/M phases, accompanied by a concomitant increase in the G1 phase, compared to miRVector LSC (Figure 2E, *Online Supplementary Figure S3*). Furthermore, miR-30e-overexpressing LSC also showed heightened levels of apoptosis compared to miRVector-overexpressing LSC (Figure 2F). To directly evaluate the effect of miR-30e overexpression on the frequency of LSC, we conducted a limiting dilution

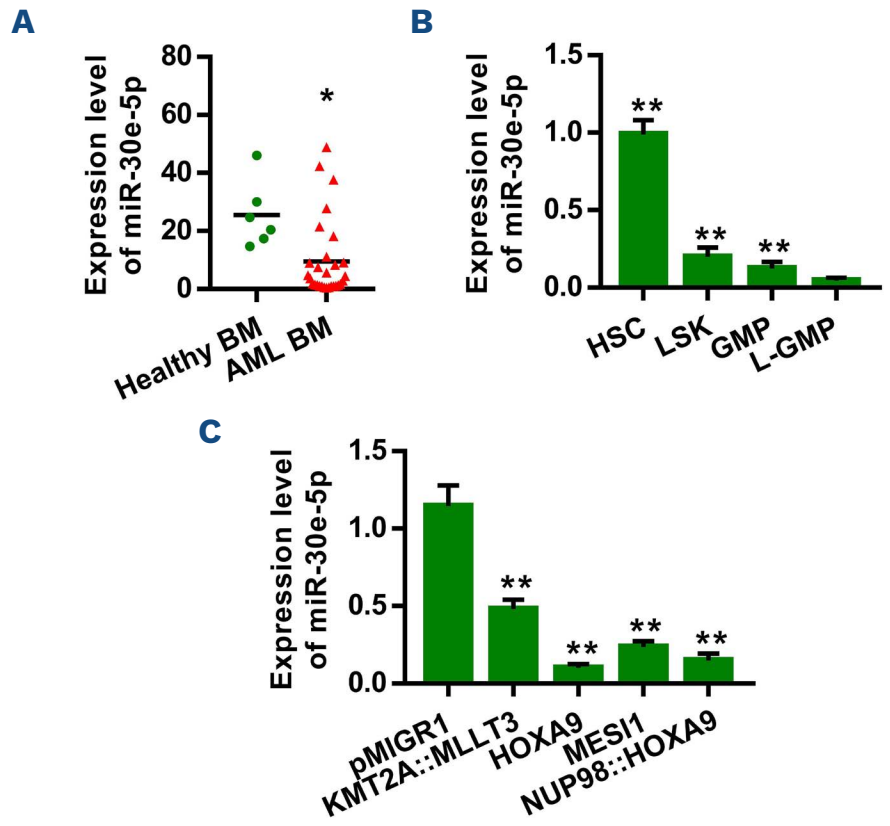

Figure 1. miR-30e-5p is repressed in both human and mouse acute myeloid leukemia cells. (A) Quantitative polymerase chain reaction (qPCR) analysis of the expression of miR-30e-5p between bone marrow cells of healthy controls and acute myeloid leukemia (AML) patients. Results are normalized to *U6* expression and expressed relative to miR-30e-5p expression in the healthy group (healthy patients, N=6; AML patients, N=29). (B) qPCR analysis of the expression of endogenous miR-30e-5p in mouse HSC, LSK, GMP, and L-GMP. The indicated cells were sorted by flow cytometry. Results are normalized to *U6* expression and expressed relative to miR-30e-5p expression in the L-GMP group (N=3). (C) qPCR analysis of the expression of endogenous miR-30e-5p in mouse hematopoietic stem and progenitor cells transduced with the retroviral vector pMIGR1 only or retrovirus expressing *KMT2A::MLLT3*, *HOXA9*, *MESI1*, *NUP98::HOXA9*. Results are normalized to *U6* expression and expressed relative to miR-30e-5p expression in the retroviral empty vector group (N=3). All data are represented as mean ± standard deviation. Two-tailed Student *t* tests were used to assess statistical significance (*$P<0.05$; **$P<0.01$). BM: bone marrow; HSC: hematopoietic stem cells ($Lin^-Sca\text{-}1^+c\text{-}Kit^+CD150^+CD48^-$); LSK cells ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$); GMP: granulocyte-monocyte progenitors ($Lin^-c\text{-}Kit^+CD34^+CD16/32^+$); L-GMP: leukemia stem cell-enriched granulocyte-monocyte progenitors ($GFP^+Lin^{low}c\text{-}Kit^+CD34^+CD16/32^+$) (N=3).

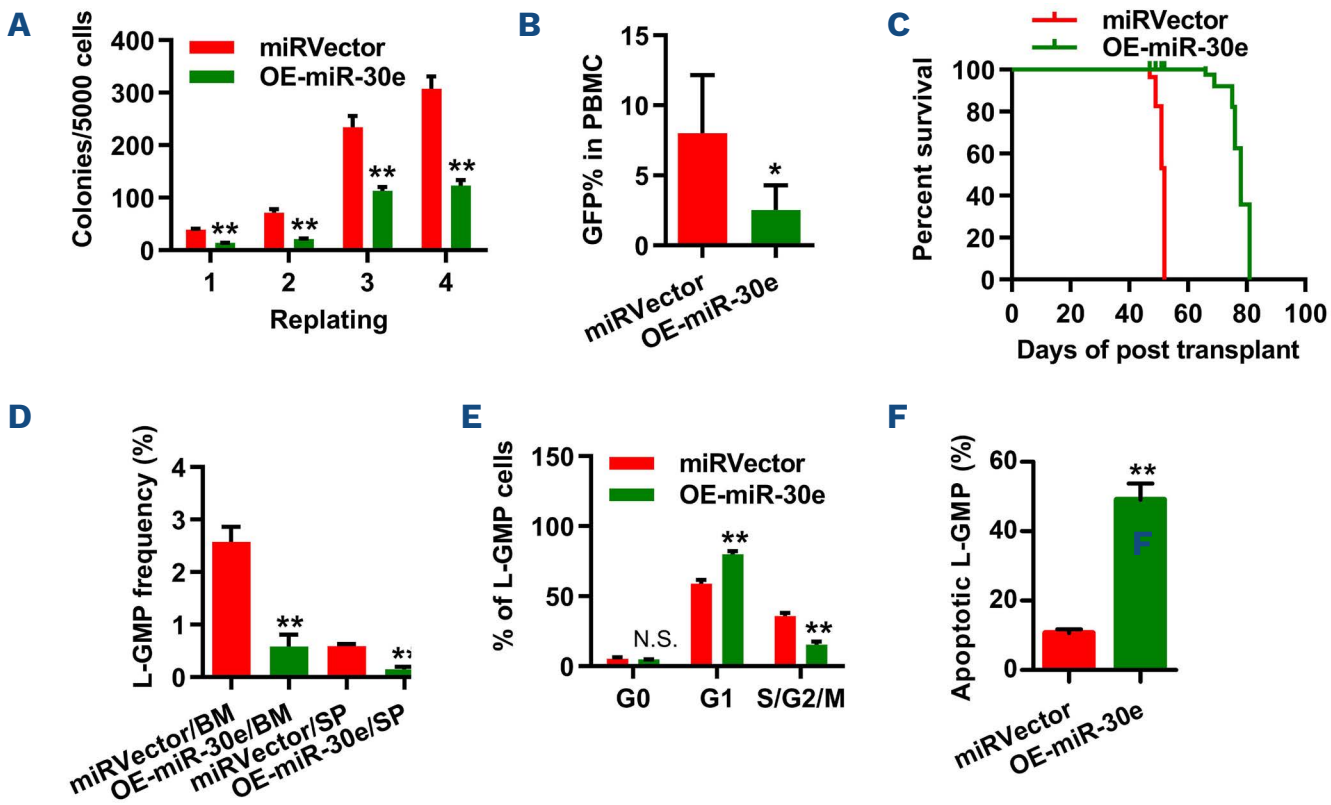

Figure 2. Forced expression of miR-30e impairs leukemia stem cell self-renewal and delays the onset of *KMT2A::MLLT3* leukemia. (A) Colony-forming assay of miRVector- or miR-30e-overexpressing acute myeloid leukemia (AML) cells (N=3). (B) Percentage green fluorescent protein positive cells in the peripheral blood at week 5 after primary bone marrow transplant with $5x10^4$ AML cells (N=6). (C) Survival analysis of primary recipient mice. The post-transplant median survival was 52 *versus* 78 days for primary recipients of miRVector- or miR-30e-overexpressing AML cells, respectively ($P<0.01$, Mantel-Cox test, N=7). (D) Frequency of leukemia stem cell-enriched granulocyte-monocyte progenitors (L-GMP) in the bone marrow (BM) and spleen from primary recipients injected with $5x10^4$ miRVector- or miR-30e-overexpressing AML cells (N=5). (E) Cell cycle phase distribution of L-GMP cells in BM from primary recipients of $5x10^4$ with miRVector- or miR-30e-overexpressing AML cells at week 5 after transplantation (N=4). (F) Percentage of apoptotic L-GMP cells in the BM from primary recipients (N=5). Data are representative of two or three independent experiments. Excluding survival analysis, all data are represented as mean ± standard deviation. Two-tailed Student *t* tests were used to assess statistical significance (N.S.: not significant; *$P<0.05$; **$P<0.01$). miRVector: control; OE-miR-30e: cells with miR-30e overexpression; GFP: green fluorescent protein; PBMC: peripheral blood mononuclear cells; SP: spleen.

assay. As expected, the estimated LSC frequency in the miR-30e-overexpressed group was 17-fold lower than that in the miRVector-group (1/529.9 *vs.* 1/31.1) (*Online Supplementary Figure 2H*, *Online Supplememtary Table S3*). Considering that the survival disparity between miRVector- and miR-30e-overexpressing AML recipients might be partly attributed to differences in homing efficiency, we determined the homing capability of secondary transplanted miRVector- and miR-30e-overexpressing AML cells. The results showed that homing efficiency was similar for both miRVector- and miR-30e-overexpressing AML cells at 16 h after BM transplantation (*Online Supplementary Figure S2I*). Taken together, these data demonstrated that miR-30e impaired cell cycle and enhanced apoptosis in LSC, thereby delaying AML progression *in vivo*.

Since the miR-30e locus contains two mature miRNA (i.e., miR-30e-5p and miR-30e-3p), we next determined which mature miRNA affects AML colony formation. To this end, we transfected corresponding mature miRNA mimics into *KMT2A::MLLT3* AML cells. The results revealed that overexpression of miR-30e-5p significantly suppressed AML colony formation. In contrast, forced expression of miR-30e-3p did not have a noticeable effect on colony formation (*Online Supplementary Figure S2J*). These findings suggest that miR-30e-5p, rather than miR-30e-3p, plays a pivotal regulatory role in *KMT2A::MLLT3*-driven leukemogenesis.

Cyb561 is a direct target of miR-30e-5p in *KMT2A::MLLT3*-driven leukemia stem cells

To gain insights into the underlying mechanisms by which miR-30e regulates the self-renewal of LSC, we sorted miRVector- and miR-30e-overexpressing LSC from two groups of recipients with leukemia driven by *KMT2A::MLLT3*. We assessed the gene expression profiles of these cells using RNA-sequencing analysis (*Online Supplementary Figure S4A*). We found that 689 genes were upregulated, and 531 genes were downregulated by >2.0-fold ($P<0.01$) in miR-30e-overexpressing LSC compared with LSC expressing miRVector (*Online Supplementary Table S4*). Through gene set enrichment analysis (GSEA), we found that the downregulated genes in miR-30e-overexpressing LSC were associated with electron transport chain, oxidative phosphorylation, and glutathione metabolism (Figure 3A, *Online Supplementary Figure S4B*, *Online Supplementary Table S5*). Further analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways revealed that these differentially expressed genes could be classified into five functional groups, with more than 150 genes involved in

signal transduction, including the MAPK pathway (*Online Supplementary Figure S5*, *Online Supplementary Table S6*). Considering that miRNA typically inhibit the translation of their mRNA by binding with the 3'UTR of their direct targets, we focused on the downregulated genes. Using the miRbase online database (*www.mirbase.org*) and qPCR, we validated seven genes as the most likely direct targets of miR-30e-5p, namely *Dock7*, *Six1*, *Hoxa11*, *Cyb561*, *Six4*, *Prickle1*, and *Ikzf2* (Figure 3B, *Online Supplementary Table S7*). Among these seven miR-30e-5p-regulated genes, some (*Ikzf2*, *Six1*, and *Hoxa11*) have been shown to play important roles in LSC and AML.[18-20] To confirm the true target of miR-30e-5p in LSC, we cloned the DNA fragment covering these genes' 3'UTR into pGL3-control luciferase vector. Subsequently, we performed a luciferase reporter assay to validate this bioinformatic prediction. Our data revealed that miR-30e-5p inhibited the luciferase activity of the wild-type *Cyb561* 3'UTR construct by 45%, whereas

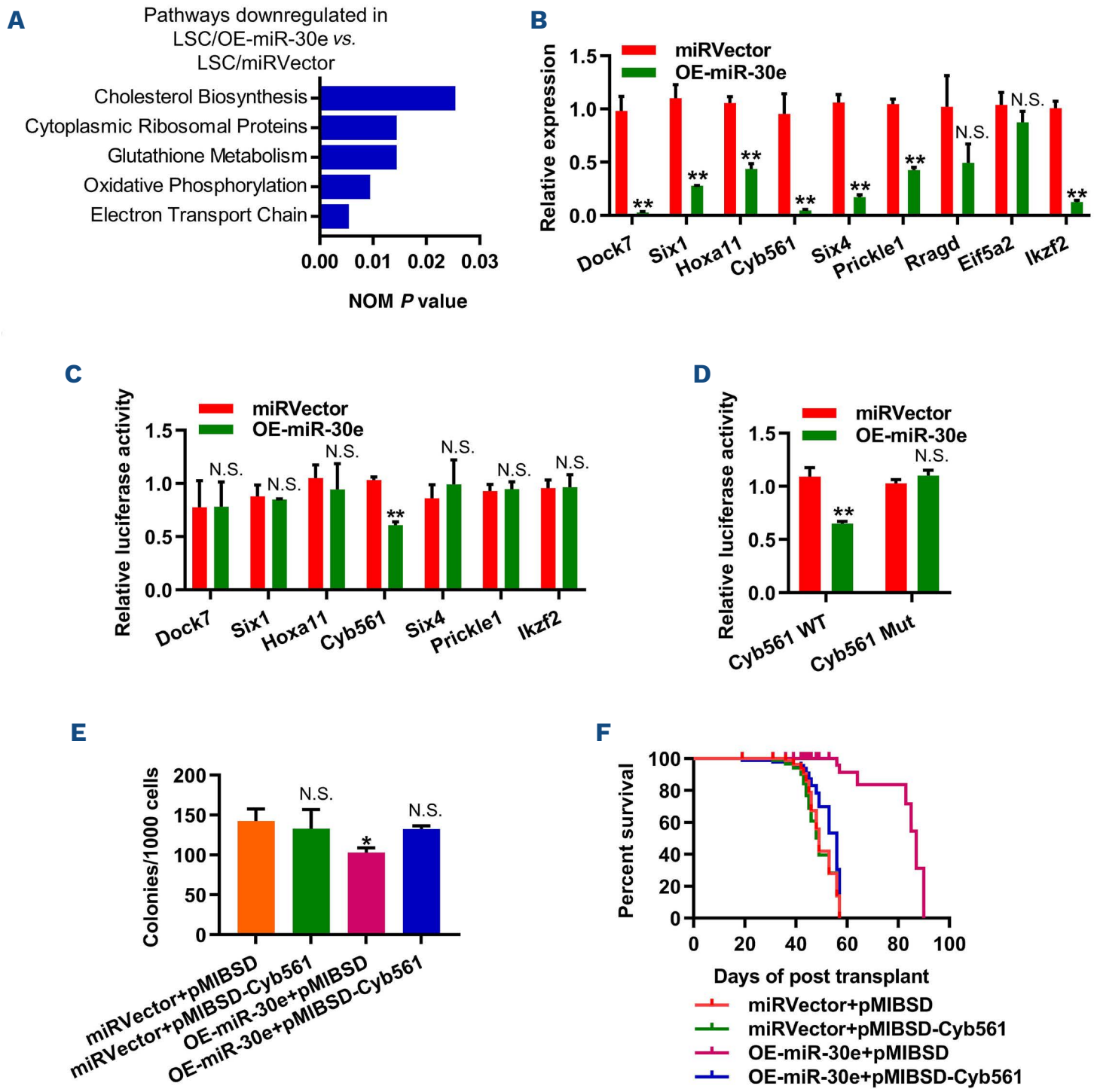

Figure 3. RNA-sequencing analysis identifies potential targets of miR-30e-5p in L-GMP cells from *KMT2A::MLLT3*-driven leukemia. (A) Enrichment plot of downregulated gene sets in miR-30e-overexpressing leukemia stem cells (LSC), as determined by gene set enrichment analysis. RNA-sequencing data for 27,359 transcripts were used for the analysis. (B) Quantitative polymerase chain reaction analysis of candidate target genes in LSC-enriched granulocyte-monocyte progenitors (L-GMP) sorted from recipients transplanted with miRVector- or miR-30e-overexpressing acute myeloid leukemia cells. Results are normalized to *Hprt* expression and expressed relative to the expression of target genes in miRVector L-GMP (N=3). (C) Luciferase reporter assay to identify the true target of miR-30e-5p (N=3). (D) *Cyb561* 3'UTR luciferase reporter assay to identify the binding site of miR-30e-5p (N=3). (E) Overexpression of *Cyb561* impaired the suppressive function of miR-30e on LSC (N=3). (F) Forced expression of *Cyb561* impeded the prolonged survival of miR-30e on *KMT2A::MLLT3*-driven leukemia (N=7). Data are representative of two or three independent experiments. All data are represented as mean ± standard deviation. Two-tailed Student *t* tests were used to assess statistical significance (N.S.: not significant; *P<0.05; **P<0.01). miRVector: control; OE-miR-30e: cells with miR-30e overexpression; NOM: nominal; WT: wild-type; mut: mutated.

the mutation in the binding site fully restored luciferase activity (Figure 3C, D, *Online Supplementary Figure S6A*). Additionally, the forced expression of miR-30e decreased the protein level of Cyb561 in mouse AML cells (*Online Supplementary Figure S6B*). These findings demonstrate that Cyb561 is a direct downstream target of miR-30e-5p in LSC. Consequently, a key question arises: does miR-30e-5p regulate LSC self-renewal through *Cyb561*? To address this question, we generated retroviral particles expressing *Cyb561* and transduced the miRVector- and miR-30e-overexpressing AML cells. The expression level of *Cyb561* was upregulated 9.86-fold in miRVector/pMIBSD-Cyb561 AML cells compared with miRVector/pMIBSD AML cells (*Online Supplementary Figure S7A*). Overexpression of *Cyb561* rescued the colony-forming capacity of forced-expressed miR-30e LSC *in vitro* (Figure 3E). Furthermore, functional assays on LSC revealed that the overexpression of *Cyb561* attenuated the effects of miR-30e on leukemia progression by regulating the cell cycle and apoptosis of LSC (Figure 3F, *Online Supplementary Figure S7B-D*). The limiting dilution assay showed that overexpression of *Cyb561* resulted in an approximately 3.80-fold increase in LSC frequency (1/124.7) in AML mice overexpressing miR-30e compared to AML mice transplanted with overexpressing-miR-30e/pMIBSD-AML cells (1/474.3) (*Online Supplementary Table S8*). Together, these findings demonstrate that *Cyb561* is a direct functional target of miR-30e-5p in LSC.

Cyb561 deficiency delays the development of *KMT2A::MLLT3*-driven acute myeloid leukemia by miR-30e-5p/ROS signaling pathway

Since miR-30e-5p suppressed the expression of endogenous *Cyb561*, we aimed to determine whether the knockdown of endogenous *Cyb561* could impair AML development. To address this issue, we knocked down *Cyb561* in *KMT2A::MLLT3*-driven LSC by inducible lentivirus-expressing shRNA. Our data showed that *Cyb561* shRNA #1, #2 and #3 reduced the expression of *Cyb561* in mouse AML cells by 57%, 80% and 31%, respectively (*Online Supplementary Figure S8A*). Subsequently, we conducted cell growth and colony-forming/replating assays using *KMT2A::MLLT3*-driven LSC transduced with scrambled shRNA (control) and two *Cyb561* shRNA (#1 and #2). Knockdown of *Cyb561* significantly inhibited the cell growth and reduced colony numbers compared to the control group (*Online Supplementary Figure S8B, C*). We then transplanted an equal number of shRNA-transduced LSC into irradiated recipients. Three to 5 weeks after BM transplantation, we analyzed AML cells in peripheral blood, spleen weight, LSC frequency, cell cycle, and apoptosis of LSC. The results showed that inhibition of *Cyb561* decreased the frequency of GFP^+ AML cells in peripheral blood, spleen weight, and LSC frequency in BM compared with the control group (Figure 4A, *Online Supplementary Figure S8D, E*). Suppression of *Cyb561* expression also attenuated the cell cycle and promoted apoptosis of LSC (Figure 4B, C, *Online Supplementary Figure S8F*). The limiting dilution assay revealed that knockdown of endogenous *Cyb561* resulted in an 11.8-fold reduction in LSC frequency (1/335.3) in AML mice transplanted with *Cyb561* shRNA #2-AML cells compared to AML mice transplanted with shRNA control (1/28.4) (*Online Supplementary Table S9*). It has been reported that CYB561 is a type of transmembrane protein consisting of six transmembrane helices and two b-type hemes on each side of the membrane, and it plays a role in the ascorbate recycling process as dehydroascorbate (DHA) reductase.[21] Ascorbate oxidation/reduction is closely associated with ROS homeostasis.[22] This suggests a possible link between CYB561 and intracellular ROS, which may be directly or indirectly involved in the cell cycle and apoptosis. Therefore, we investigated whether the knockdown of *Cyb561* affects ROS production. Mean fluorescence intensity analysis revealed elevated intracellular ROS levels in *Cyb561*-knockdown LSC (*Online Supplementary Figure S8G*). Next, we examined the effect of miR-30e on the level of ROS in LSC from the recipients. As expected, overexpression of miR-30e increased intracellular ROS levels in LSC. However, overexpression of *Cyb561* reduced intracellular ROS levels in ectopic miR-30e-expressing LSC (*Online Supplementary Figure S7E, F*). To further investigate whether miR-30e/*Cyb561* impaired LSC by regulating intracellular ROS, we used the ROS scavenger NAC and Tiron to decrease ROS levels in the AML cells. Both NAC and Tiron rescued the colony-forming capacity of LSC overexpressing miR-30e and LSC with *Cyb561* knockdown (*Online Supplementary Figures S9A* and *S10A*). LSC functional assays, showed that NAC treatment impaired the roles of miR-30e and *Cyb561* in LSC and leukemia progression (Figure 4D, E, *Online Supplementary Figures S9B-D* and *S10B-D*). Given that CYB561 acts as a reductase in the ascorbate-DHA recycling process and there is an enriched gene set (i.e., glutathione metabolism) in LSC according to GSEA, we tested whether the miR-30e/*Cyb561*/ROS axis affects the levels of endogenous glutathione, ascorbate and DHA in AML cells. The results revealed a decrease in the levels of glutathione and ascorbate accompanied by an increase in DHA levels in AML cells overexpressing miR-30e and in AML cells with *Cyb561* knockdown (*Online Supplementary Figure S11*). Furthermore, we examined the functions of miR-30e and *Cyb561* in normal HSC, given the intriguing findings in LSC. As shown in *Online Supplementary Figure S12*, neither overexpression of miR-30e nor knockdown of *Cyb561* influenced the repopulation capacity of normal HSC. Collectively, our data indicate that miR-30e-5p functions as a negative regulator of the *Cyb561*-ROS signaling pathway (Figure 4F).

Overexpression of *Homo sapiens*-miR-30e and knockdown of *CYB561* impairs human acute myeloid leukemias

Although the results from the murine models provided robust evidence for the critical role of miR-30e-5p and

Cyb561 in the initiation and maintenance of murine LSC induced by *KMT2A::MLLT3*, it remained unclear whether miR-30e-5p or *Cyb561* also affects human leukemia. To address this question, we generated retroviral particles expressing *Homo sapiens* (hsa)-miR-30e and transduced them into six different human leukemic cells, namely THP-1, MONOMAC-6, NOMO-1, Kasumi-1, NB4, and human MA9.3 cells. Cell growth/proliferation assays demonstrated that forced expression of hsa-miR-30e inhibited the growth of all human leukemic cells (Figure 5A-D, *Online Supplementary Figure S13A-C*). Furthermore, overexpression of miR-30e induced extensive apoptosis in human AML cells without affecting their differentiation (*Online Supplementary Figure S13D-F*). *In vitro* colony-forming assays revealed reduced colony numbera in the hsa-miR-30e-overexpressing groups in comparison to the miRVector-expressing groups (Figure 5E). Moreover, ectopic expression of hsa-miR-30e suppressed the expression level of *CYB561* in human AML cells (Figure 5F). To further investigate whether miR-30e exerted similar effects on the functions of human LSC, we analyzed the cell cycle and apoptosis of human LSC ($CD34^+CD38^-$) in human MA9.3 cells, which closely resemble AML cell models containing the *KMT2A::MLLT3* fusion found in patients' AML samples.[15] As depicted in *Online Supplementary Figure S13G-H*, overexpression of miR-30e increased the G1 phase while decreasing the S/G2/M phases of LSC and promoted the apoptosis of LSC. To assess the relevance of CYB561 in human AML cells, we first examined the clinical significance of CYB561 expression levels in AML patients and the normal human population. The results showed that *CYB561* expression was upregulated in BM mononuclear cells of FAB subtypes AML-M1, M2, M3, M4, M5 compared to normal BM monocytes (Figure 6A). Additionally, CYB561 expression was increased in LSC from AML patients compared to normal GMP (Figure 6B). Notably, AML patients with higher levels of

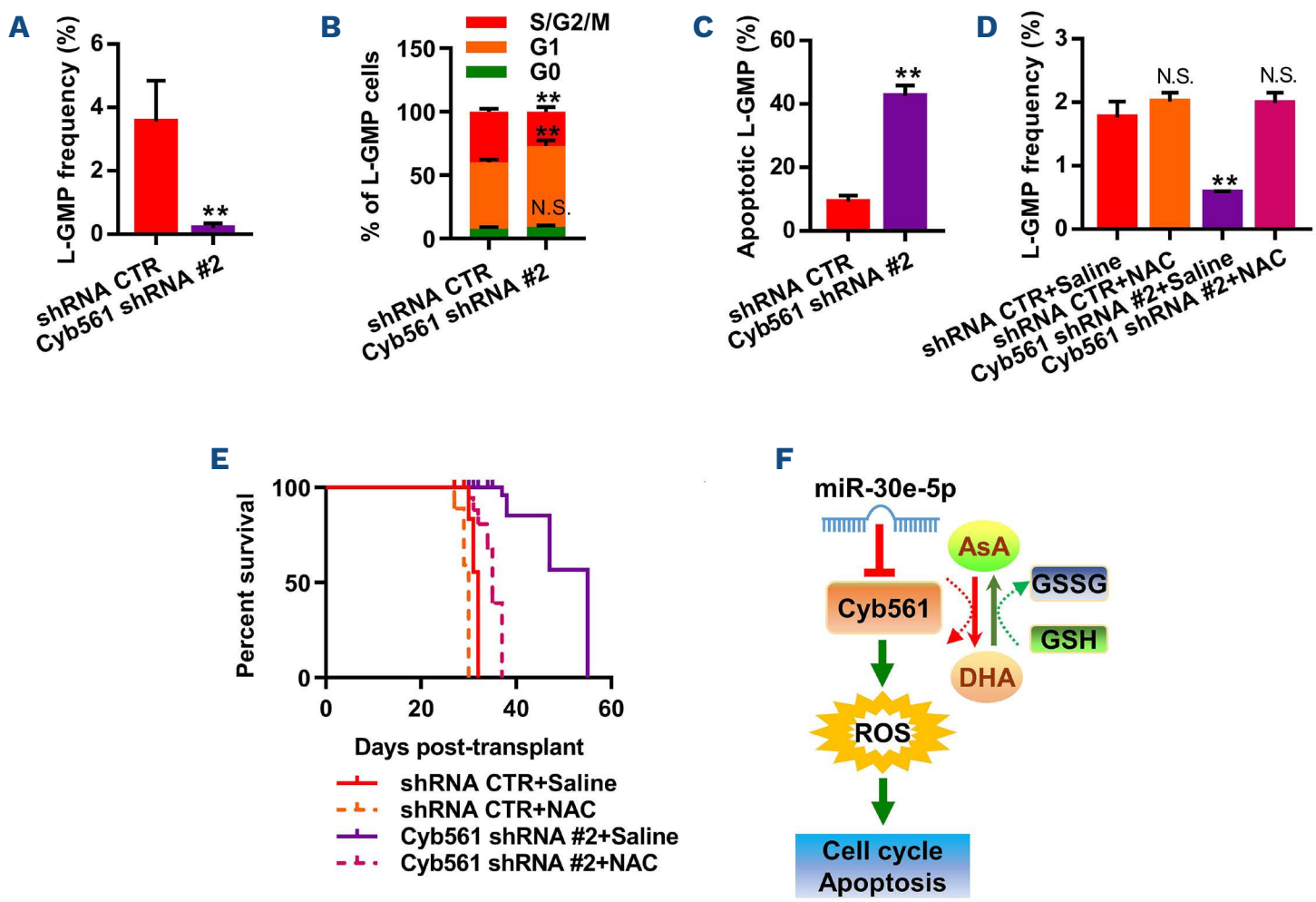

Figure 4. Knockdown of *Cyb561* diminishes self-renewal of *KMT2A::MLLT3*-driven leukemia stem cells by enhancing intracellular levels of reactive oxygen species. (A) Frequency of leukemia stem cell-enriched granulocyte-monocyte progenitors (L-GMP) in the bone marrow (BM) from primary recipients injected with $1x10^5$ acute myeloid leukemia (AML) cells transduced with control short hairpin (sh) RNA (shRNA CTR) or *Cyb561* shRNA #2, at week 4 after transplantation (N=5). (B) Cell cycle phase distribution of L-GMP cells in BM from primary recipients injected with shRNA CTR or *Cyb561* shRNA #2 AML cells, at week 5 after transplantation (N=4). (C) Percentage of apoptotic L-GMP cells in the BM from primary recipients transplanted with shRNA CTR or *Cyb561* shRNA #2 AML cells (N=4). (D) Frequency of L-GMP in the BM from recipients receiving AML cells transduced with shRNA CTR or *Cyb561* shRNA #2 following treatment with saline or N-acetyl-L-cysteine (NAC) (N=5). (E) Survival analysis of recipient mice receiving AML carrying shRNA CTR or *Cyb561* shRNA #2 after treatment with saline or NAC. The median survival of the four groups of recipients was 32, 30, 55, and 35 days after transplantation ($P<0.01$, Mantel-Cox test, N=6). (F) Diagrammatic model of the miR-30e-5p/*Cyb561*/ROS signaling pathway. Data are representative of two or three independent experiments. All data are represented as mean ± standard deviation. Two-tailed Student *t* tests were used to assess statistical significance (N.S.: not significant; *$P<0.05$; **$P<0.01$). AsA: ascorbate; GSSG: oxidated glutathione; GSH: reduced glutathione; DHA: dehydroascorbate; ROS: reactive oxygen species.

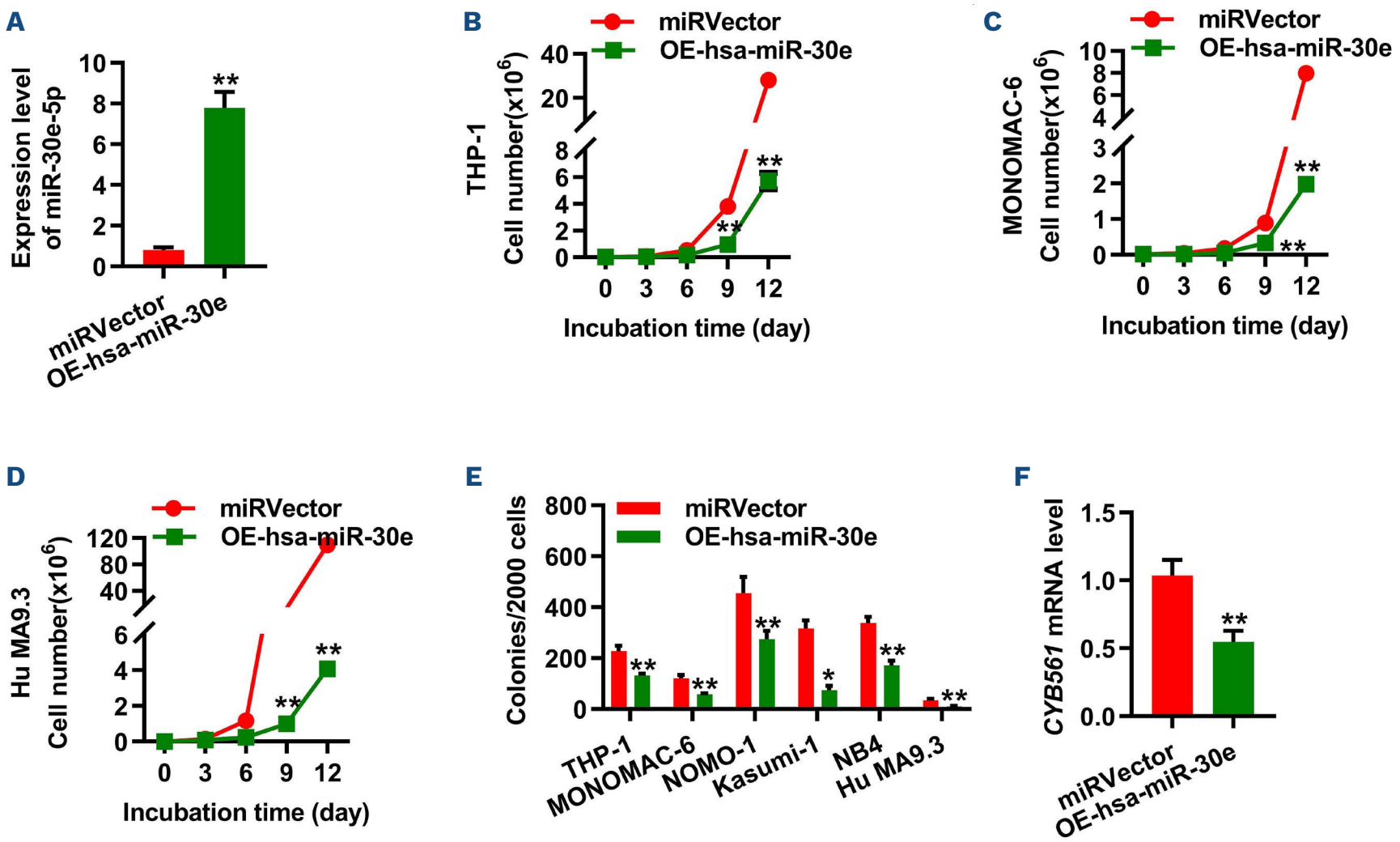

Figure 5. Overexpression of miR-30e impairs human acute myeloid leukemia cell growth. (A) Quantitative polymerase chain reaction (qPCR) analysis of miR-30e-5p expression in THP-1 cells. Results are normalized to *U6* expression and expressed relative to miR-30e-5p expression in the miRVector group (N=3). (B-D) Ectopic expression of miR-30e suppresses the growth of THP-1 (B), MONOMAC-6 (C), and Hu MA9.3 (D) cells (N=3). (E) Colony-forming assay of human leukemia cells by overexpressed-miR-30e (N=3). (F) qPCR analysis of the expression level of *CYB561* in THP-1 cells transduced with miRVector or miR-30e. Results are normalized to *GADPH* expression and expressed relative to *CYB561* in the miRVector group (N=3). Data are representative of two or three independent experiments. All data are represented as mean ± standard deviation. Two-tailed Student *t* tests were used to assess statistical significance (*P<0.05; **P<0.01). miRVector: control; OE-hsa-miR-30e: cells with *Homo sapiens* miR-303 overexpression.

CYB561 expression displayed a significantly shorter overall survival time, as observed through gene expression profiling interactive analysis (GEPIA: *http://gepia.cancer-pku.cn/*) (Figure 6C). Next, we examined functional potentials of *CYB561* in human AML cells by doxycycline-inducible shRNA. As shown in Figure 7A, *CYB561* shRNA #1 and #2 effectively reduced the expression of *CYB561* in human leukemic cells by 56% and 45%, respectively. The knockdown of *CYB561* suppressed the growth of all human leukemic cells and promoted apoptosis *in vitro*, while having no impact on the differentiation of human AML cells (Figure 7B-E, *Online Supplementary Figure S14A-F*). Moreover, inhibition of *CYB561* increased the G1 phase while decreasing the S/G2/M phases of human LSC and promoted the apoptosis of human LSC (*Online Supplementary Figure S14G, H*).

To explore the therapeutic potential of targeting miR-30e-5p and CYB561 in AML, we designed mimics of hsa-miR-30e-5p and siRNA corresponding to the *CYB561* coding region. These molecules were delivered into various human leukemic cells. We observed inhibition of human leukemic cell growth upon treatment with these miRNA mimics and siRNA across all AML cell lines (*Online Supplementary Figures S15* and *S16*). Taken together, these findings provide compelling evidence for the promising therapeutic potential of miR-30e-5p and CYB561 in human AML.

Discussion

LSC, a rare population of AML cells, play unique roles in the initiation, maintenance, and propagation of AML. Similar to normal HSC, LSC reside in the hypoxic BM niche and their biological functions are closely related to the intracellular ROS level and oxidative stress status.[23] It has been observed that excessively elevated ROS levels can impair the self-renewal capacity of LSC.[24] Therefore, targeting ROS and ROS-associated regulators in LSC represents a promising therapeutic strategy for improving long-term outcomes in AML. In this study, we have revealed that miR-30e-5p negatively regulates LSC self-renewal mainly through its direct target Cyb561, a transmembrane protein

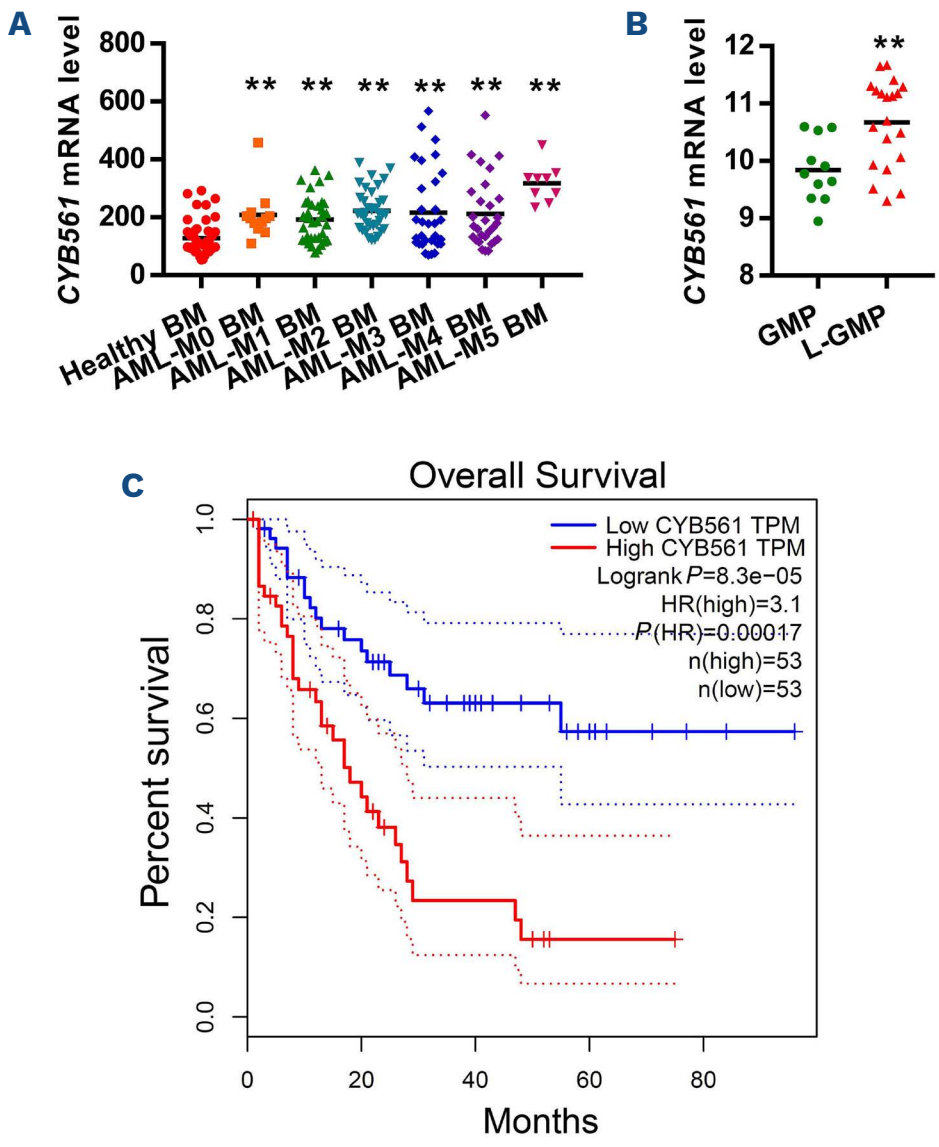

Figure 6. *CYB561* is upregulated in acute myeloid leukemia patients. (A) The difference in expression of *CYB561* between normal bone marrow cells from healthy controls and from patients with different subtypes of acute myeloid leukemia (AML) according to the French-American-British classification. The data were obtained from a public microarray database (access numbers: GSE11504, GSE19429, GSE10358) (normal patients, N=42; M0 patients, N=12; M1 patients, N=35; M2 patients, N=36; M3 patients, N=31; M4 patients, N=29; M5 patients, N=9). (B) The expression of *CYB561* between normal granulocyte-monocyte progenitors (GMP) and leukemia stem cell-enriched granulocyte-monocyte progenitors (L-GMP) from patients with AML (normal patients, N=11; AML patients, N=20). (C) Correlation analysis between *CYB561* expression level and overall survival in AML patients according to GEPIA. BM: bone marrow; FAB: French-American-British; TPM: transcripts per million; HR: hazards ratio.

that functions as a reductase for regenerating ascorbate and providing reduced iron for transmembrane transport.[21] The functional roles of *Cyb561* in leukemogenesis have not been previously reported. However, in our investigations, we observed that miR-30e-5p/*Cyb561* modulates LSC self-renewal by activating intracellular ROS. Intriguingly, both forced expression of miR-30e-5p and inhibition of *Cyb561* have shown the ability to delay leukemia progression, suggesting that both miR-30e-5p and *Cyb561* possess potential therapeutic effects on LSC in AML.

miR-30e-5p is expressed at a high level in normal HSC and plays a significant role in various crucial biological processes. Previous studies have demonstrated its potential as a tumor suppressor in different types of cancer, such as bladder cancer, nasopharyngeal carcinoma, and liver cancer, and induces apoptosis of chronic myeloid leukemia cells.[9,13,25,26] The level of expression of miR-30e-5p is elevated in patients with acute lymphoblastic leukemia and reduced in patients with chronic myeloid leukemia.[13,16] Despite these notable findings regarding miR-30e-5p in cancer development and leukemia, to the best of our knowledge, there is no existing evidence demonstrating that overexpression of miR-30e-5p not only delays the onset of AML and targets LSC by regulating the cell cycle and apoptosis *in vivo*, but also acts as a negative regulator of the transmembrane reductase CYB561.

CYB561 is a di-heme transmembrane protein that plays a crucial role in ascorbate recycling and iron homeostasis and is associated with cellular senescence.[21,27] It is expressed highly in metabolically active human tissues, such as the brain, kidney, and heart.[28] Despite its clear physiological significance, the functions of *Cyb561* in animal cells, particularly in LSC, remain unexplored. Previous studies have indicated that CYB561 mutations in patients result in a novel orthostatic hypotension syndrome, and that CYB561 could serve as a potential prognostic biomarker for breast cancer.[28,29] In our current study, we found that miR-30e-5p downregulates the expression of *Cyb561* in LSC. Intriguingly, the knockdown of *Cyb561* in *KMT2A::MLLT3*-driven LSC prolongs the survival of recipient mice with a reduced frequency of LSC. Furthermore, we observed a negative correlation between overall survival and *CYB561* expression level in AML patients. These findings suggest that *Cyb561* acts as a potential suppressor of AML initiation and development and could serve as a biomarker for leukemia prognosis.

Through GSEA of downregulated gene sets in miR-30e-overexpressed LSC, we identified significant enrichment of gene sets associated with the electron transport chain, oxidative phosphorylation, and glutathione metabolism. These gene sets are highly relevant to intracellular ROS production and balance. Glutathione, as the most abundant endogenous antioxidant in mammalian cells, plays a crucial role in maintaining a “non-toxic” level of ROS.

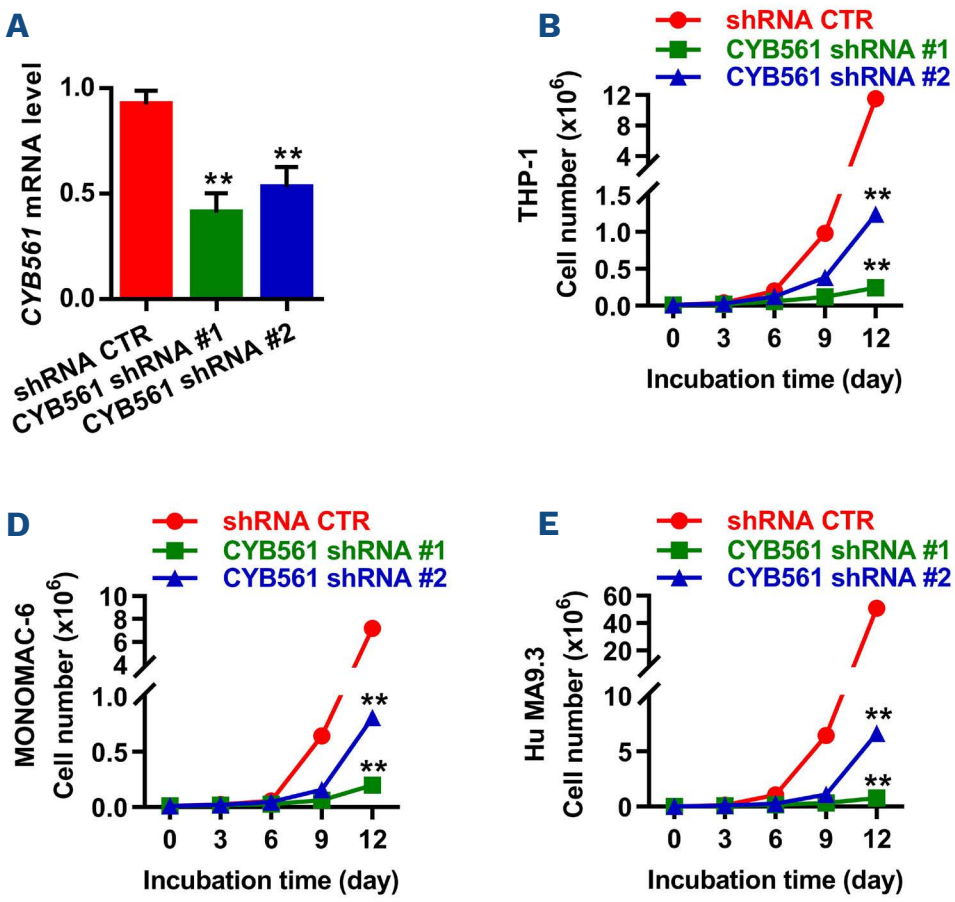

Figure 7. Knockdown of *CYB561* suppresses the growth of human acute myeloid leukemia cells. (A) Quantitative polymerase chain reaction analysis of inducible *CYB561* knockdown in NB4 cells. NB4 cells were transduced with lenitiviruses containing a scrambled short hairpin (sh) RNA control (CTR), or inducible *CYB561* shRNA. After puromycin selection, 1 μg/mL of doxycycline was added to the medium for 72 h. Results are normalized to *HPRT* expression and expressed relative to the shRNA control group (N=3). (B-E) Knockdown of *CYB561* suppresses the growth of THP-1 (B), NOMO-1 (C), MONOMAC-6 (D), and Hu MA9.3 (E) cells (N=3). Data are representative of two or three independent experiments. All data are represented as mean ± standard deviation. Two-tailed Student *t* tests were used to assess statistical significance (**P<0.01).

Previous studies have demonstrated a significant reduction in glutathione levels in primary human CD34$^+$ AML cells compared to CD34$^+$ normal BM cells. Consistent with these findings, our study revealed a decrease in endogenous glutathione levels in miR-30e-overexpressing LSC, which was attributed to elevated levels of intracellular ROS, similar to the results observed in *Cyb561*-knockdown LSC. CYB561, functioning as a reductase, is involved in the reduction process from DHA to ascorbate, and its deficiency can disrupt this process, leading to intracellular ROS accumulation. Our data demonstrated that both overexpression of miR-30e-5p and knockdown of *Cyb561* resulted in reduced intracellular ascorbate levels, accompanied by elevated DHA levels in LSC. Impaired ascorbate recycling due to endogenous *Cyb561* knockdown consequently led to decreased glutathione levels and increased ROS levels in LSC. Integrating these findings with our results, we propose that miR-30e-5p regulates LSC self-renewal through the Cyb561/ROS signaling pathway, involving intracellular ascorbate/glutathione metabolism. In summary, our study unveils a previously unknown miR-30e-5p/*Cyb561* axis that finely modulates an intracellular ROS signaling pathway in LSC. Based on our findings, pharmacological targeting of LSC through manipulation of miR-30e-5p or *Cyb561*, in combination with chemotherapy, holds great potential as a highly effective strategy to enhance therapeutic regimens.

Disclosures

No conflicts of interest to disclose.

Contributions

ZZ and WSW were responsible for the design of the experiments, data analysis, and manuscript preparation. YG, MH, YZ, JW, and LL performed experiments. HZ and YS contributed to the bioinformatics analysis.

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Data-sharing statement

Data that support the findings of this study are available from the corresponding author upon reasonable request.

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