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LINC00504 promotes the progression of acute myeloid leukemia by targeting MDM2

Jin-Hua YAN[1,*], Ling YAO[2], Ying TAN[3], Yue WANG[1]

[1]Department of Hematology and Rheumatology, The First Hospital of Nanchang, Nanchang, Jiangxi, China; [2]Department of Gastroenterology, The First Hospital of Nanchang, Nanchang, Jiangxi, China; [3]Department of Gerontology, The First Hospital of Nanchang, Nanchang, Jiangxi, China

**Correspondence: yanjinhua269@163.com*

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Acute myeloid leukemia (AML) is a highly heterogeneous hematopoietic malignant tumor, accompanied by the abnormal cloning of myeloid hematopoietic stem cells, little is known about its etiological role and pathogenesis. We aimed to explore the effect and regulatory mechanism of LINC00504 on the malignant phenotypes of AML cells. In this study, LINC00504 levels in AML tissues or cells were ascertained by PCR. RNA pull-down and RIP assays were conducted to verify the combination of LINC00504 and MDM2. Cell proliferation was detected by CCK-8 and BrdU assays, apoptosis was checked by flow cytometry, and glycolytic metabolism levels were detected by ELISA analysis. The expressions of MDM2, Ki-67, HK2, cleaved caspase-3, and p53 were detected by western blotting and immunohistochemistry. A xenograft tumor model was used to detect the role of LINC00504 *in vivo*. Results showed that LINC00504 was highly expressed in AML and its high expression was related to clinicopathological features in AML patients. LINC00504 knockdown significantly inhibited the proliferation and glycolysis, while inducing apoptosis of AML cells. Meanwhile, LINC00504 downregulation also exerted a significant alleviating effect on AML cell growth *in vivo*. In addition, LINC00504 could bind to MDM2 protein and positively regulate its expression. Overexpression of LINC00504 promoted the malignant phenotypes of AML cells and partially reversed the inhibitory effects of LINC00504 knockdown on AML progression. In conclusion, LINC00504 facilitated AML cell proliferation and suppressed apoptosis through upregulating MDM2 expression, suggesting that LINC00504 may serve as a prognostic marker and therapeutic target in patients with AML.

Key words: acute myeloid leukemia; LINC00504; MDM2

Acute myeloid leukemia (AML) is a common malignant tumor of the hematopoietic system, accounting for ~70% of acute leukemia, and has become the most common type of adult leukemia [1]. During the onset of AML, a large number of abnormal proliferative primordial and naïve cells in bone marrow could inhibit normal hematopoiesis, which is characterized by bleeding, anemia, infection, and cellular infiltration [2]. At present, although chemotherapy is the main treatment modality for AML, the overall 5-year survival rate for the majority of AML patients after receiving chemotherapy is still dismal, less than 40% [3]. Additionally, due to the toxic side effects of chemotherapeutic drugs, it is urgent to explore a new safe and effective treatment modality. Thus, it is still necessary to further explore the mechanism for the occurrence and development of AML.

Long non-coding RNA (lncRNA) has been demonstrated to participate in the normal physiological process of the body and is even involved in the pathological processes of various diseases including AML [4]. For instance, lncRNA CCAT1 was found to be highly expressed in AML patient samples and regulated the growth and differentiation of AML cells as a competitive endogenous RNA (ceRNA) [5]. Moreover, upregulation of lncRNA PANDAR could predict the poor prognosis of AML patients, suggesting that PANDAR may become a potential diagnostic marker of AML [6]. As a widely studied oncogene, LINC00504 located on human chromosome 8, plays a pro-oncogenic function in multiple tumors, including non-small cell lung cancer [7] and breast cancer [8]. It is worth noting that a recent study reported that the high expression of LINC00504 in the sera of AML patients based on TCGA database was negatively correlated with the worse overall survival of AML patients [9]. However, there are no more studies to validate the specific role of LINC00504 in AML, especially the potential regulatory mechanisms.

Murine double minute-2 (MDM2), an oncoprotein that functions as the inhibitor of p53, plays an important role in a variety of diseases. In most AML patients, MDM2 tended to be overexpressed, and strongly enhanced the anti-apoptosis ability of leukemic cells by inhibiting p53, which led to chemotherapy resistance and disease recurrence [10, 11]. Although MDM2 has been confirmed to serve an essential role in AML progression, its potential regulatory mechanism, especially the interaction with lncRNAs remains to be further explored.

In this study, we first examined the expression patterns of LINC00504 and MDM2 in AML tissues or cells. Then, through a variety of *in vivo* and *in vitro* experiments, including proliferation, apoptosis, and xenograft tumor, we verified the role of LINC00504 in promoting AML progression by regulating MDM2. Our results are expected to provide new ideas for the treatment of AML.

Patients and methods

Tissue sample collection. Bone marrow samples from 66 AML patients and normal specimens from 44 healthy donors were collected in our hospital. After bone marrow puncture, mononuclear cells were extracted and stored at −80 °C for follow-up experimental detection. This present study was based on the principles expressed in Helsinki Declaration. All the clinical experiments were approved by the Ethics Committee of The First Hospital of Nanchang (Ethics approval number: KY2020011). Participants provided written informed consent to participate in the study and consent for publication was obtained from all participants.

Cell culture and cell transfection. The normal monocyte cells (HS-5) and AML cells (HL60, THP-1, U937, and KG-1) were acquired from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cell lines were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. And all cells were kept in an incubator at 37 °C and 5% CO_2.

Short hairpin RNA (shRNA) against LINC00504 (sh-LINC00504) and its corresponding negative controls (sh-NC) were offered by Genechem (Shanghai, China). pcDNA-mediated MDM2 overexpression plasmid (MDM2) and its control (vector) were purchased by Ribobio (Guangzhou, China). AML cells (HL60 and THP-1) were transfected with these above vectors via Lipofectamine 3000 (Invitrogen, USA). RT-qPCR assay was carried out to assess the efficiency of the transfections after the 48 h incubation. Sequences for control- and target-specific shRNAs are as follows: shRNA-NC (CTCTCAACCCTTTAAATCTGA); shRNA-LINC00504#1 (GCCAGAGCTGTACCTTTGAGA); shRNA-LINC00504#2 (GAAGACAAGGTTGAAGATGAT); shRNA-LINC00504#3 (GAGCGCAGCCTTGCCTCTTAC).

qRT-PCR. Total RNAs were extracted from tissues and cell lines using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Total RNAs were reversed into cDNA using a Script cDNA synthesis kit (Cwbio, Beijing, China) and a miRNA 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). Next, the SYBR Green Master PCR mix (Takara, Dalian, China) was used for the qRT-PCR carried out in an ABI 7900 Fast Real-Time PCR System (Applied Biosystems, USA). The relative expressions were determined using the $2^{-\Delta\Delta}$Ct approach and then normalized to GAPDH. The sequences of primers were listed as follows: LINC00504 forward 5'-GTGACTCGAGCTTGCCTCT-GCCATGT-3'; LINC00504 reverse 5'-GTGAGCGGCC-GCTTTCAGAGTGAAACAATACTT-3'; MDM2 forward 5'-TGT TGGTGCACAAAAAGACACTT-3'; MDM2 reverse 5'-GCACGCCAAACAAATCTCCTA-3'; GAPDH forward 5'-ACCCAGAAGACTGTGGATGG-3'; GAPDH reverse 5'-TTCAGCTCAGGGATGACCTT-3'.

CCK-8 assay. Cell proliferation was determined via a CCK-8 assay (TransGen, Beijing, China). Briefly, collected transfected cells were transferred to a 96-well plate (5×10^3 cells/well), and let to stand for 24 h. Then, 10 μl CCK-8 was added into each well at 24, 48, and 72 h, followed by 2 h incubation for 37 °C. The optical density of every well at 450 nm was measured by an automatic microplate reader (Bio-Rad, USA).

BrdU proliferation assay. According to the manufacturer's guidelines, BrdU analyses were conducted using the BrdU proliferation kit (Millipore, USA). The optical density (450 nm) of each well was analyzed using a microplate reader (Bio-Rad, USA).

Flow cytometry. Cell apoptosis was performed using the Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich, USA). In brief, cells were collected and harvested by centrifugation at 3,000 × g for 2 min. And then, these cells were resuspended in the binding buffer. Subsequently, 5 μl of PI and 5 μl of Annexin V were added and mixed with the cell solution. After incubating for 20 min in the dark, the apoptotic cells were analyzed on a FACScan flow cytometry (BD Biosciences, USA) with BD CellQuest Pro software (version 5.1, BD Biosciences).

ELISA analysis. Glycolytic metabolism levels (glucose consumption, lactate production, and ATP level) were examined using an ELISA assay. Briefly, the glucose consumption, lactate production, and ATP level were assessed by glucose assay kit, lactate assay kit, and ATP assay kit (all purchased from Biyuntian, China), respectively.

Western blotting. Total proteins were lysed with RIPA buffer (Thermo Scientific, USA), and their concentration and quantification were determined via a BCA kit (Thermo Scientific, USA), followed by separation via 12% SDS-PAGE. Subsequently, the separated protein was transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA), followed by 2 h immersion in 5% skim milk and overnight sealing with primary antibodies [Ki-67 (1:1000, ab833,

Abcam), cleaved caspase-3 (1:1000, ab9661, Abcam), HK2 (1:1000, ab21985, Abcam), p53 (1:1000, ab32124, Abcam), MDM2 (1:1000, ab38618, Abcam), and β-actin (1:1000, ab8226, Abcam)] from Abcam company (USA). Afterward, the membrane was subjected to 1 h incubation at 37 °C with horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:5000, ab6721, Abcam). Finally, electrochemiluminescence (ECL, Thermo Fisher, USA) was adopted for development. The intensity of protein bands was detected by Image-Pro Plus 6.0 software (NIH Image, USA).

RNA pull-down assay. RNA pull-down assay was performed using the pull-down kit (Pierce, USA) according to the manufacturer's protocol. Briefly, transfected AML cells were incubated with biotinylated-LINC00504 and biotinylated NC. Cells were incubated for 48 h and then lysed in RNase-free cell lysis solution. Then these cell lysates were incubated with magnetic beads (Invitrogen, USA) overnight at 4 °C. After washing, the pellet was lysed with TRIzol reagent (Invitrogen, USA), and the binding between MDM2 and LINC00504 was measured via western blotting.

RNA immunoprecipitation (RIP) assay. Ago2 acts as a core component of protein-governed RNA-induced silencing complexes (RISC). We performed the RIP assay to determine the binding between MDM2 and LINC00504 using a Magna RIP Kit (Millipore, USA). Magnetic beads were pre-coated with anti-Ago2 or anti-IgG. Cells were lysed and then exposed to antibody-coated beads. RNA complexes bound to beads were eluted and analyzed by RT-qPCR.

***In vivo* experiment.** LINC00504-targeted short-hairpin RNA (sh-circ) or sh-NC was assembled and lentivirus-packaged by Genepharma. Twelve BALB/c mice (6-week-old; male) were provided by Vital River (Beijing, China). Mice were raised at pathogen-free conditions to acclimate for one week. Briefly, to construct tumorigenesis models in nude mice, viable THP-1 cells (2×10^6 cells/mouse) infected with sh-circ or sh-NC-related lentiviral particles were hypodermically transplanted into the right flank of mice (n=6 in each group). Tumor volume was weekly measured (length × width^2/2). After 30 d, tumor nodes were excised from mice that were euthanized with sodium pentobarbital (180 mg/kg). Tissue samples were rapidly frozen and placed at −80 °C for long-term storage. All the animal experiments were in accordance with the National Institutes of Health (NIH) guidelines. All procedures were performed with the approval of Nanchang Hospital.

Immunocytochemistry. Slices were fixed in formaldehyde and then embedded in paraffin. Antigen repair was performed and these slices were incubated with primary antibodies including Ki-67 (1:1000, ab833, Abcam), cleaved caspase-3 (1:1000, ab9661, Abcam), HK2 (1:1000, ab21985, Abcam), p53 (1:1000, ab32124, Abcam), and MDM2 (1:1000, ab38618, Abcam) at 4 °C overnight. Then these slices were incubated with the corresponding HRP-labeled second antibody (1:5000; LK-GAR007, Abcam) for 30 min. Finally, DAB was added to develop the color.

Statistical analysis. The experimental data were processed using GraphPad Prism 8 (GraphPad, USA). Data were displayed as mean ± standard deviation (SD). For difference comparison, the Student's t-test was utilized for comparison between the two groups, and one-way ANOVA followed by Tukey post hoc was utilized for comparison among multiple groups. Kaplan-Meier (K-M) curves and log-rank test were performed to exhibit and analyze the overall survival (OS) in AML patients. Spearman correlation analysis was conducted to assess the relationship between LINC00504 and MDM2. p-value <0.05 was a statistically significant difference.

Results

Upregulated LINC00504 and high LINC00504 expression associated with the poor outcome in AML. To explore the expression patterns of LINC00504 in AML, LINC00504 expression in bone marrow cells from normal and AML patients was detected by RT-PCR. Results showed that LINC00504 expression in AML patients was significantly higher than that compared to normal controls (Figure 1A).

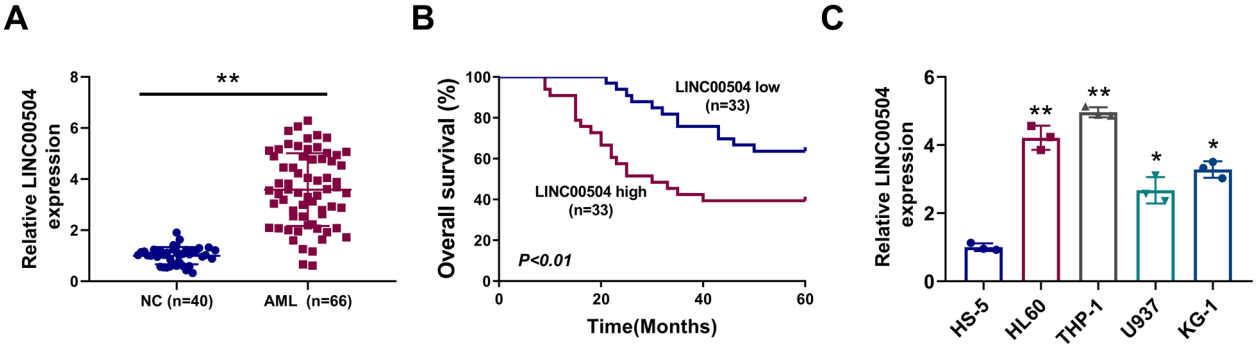

Figure 1. Upregulated LINC00504 and high LINC00504 expression are associated with poor outcome in AML. A) LINC00504 expression in AML clinical samples (n=40) and normal samples (n=66) was examined using RT-PCR. **p<0.01 vs. NC by Student's t-test. B) Correlation between LINC00504 expression with poor survival in AML patients. C) LINC00504 expression in normal bone marrow cells (HS-5) and AML cells (HL60, THP-1, U937, and KG-1) was analyzed using RT-PCR. *p<0.05 and **p<0.01 vs. HS-5 by Student's t-test. The experiments are shown as means ± SD and experiments were performed at least in duplicate.

Additionally, K-M survival analysis manifested that higher LINC00504 expression predicted a lower survival rate (Figure 1B). We also assessed the correlation between LINC00504 expression and clinical pathological features of AML patients (Table 1). Results showed that LINC00504 expression is associated with white blood cell (WBC) and cytogenetics, but not to age, gender, French-American-British (FAB) classification, or Blast in bone marrow (BM). Moreover, consistent with the LINC00504 expression detection in AML patients, the expression of LINC00504 was significantly increased in AML cells (HL60, THP-1, U937, and KG-1) compared to normal cells (HS-5) (Figure 1C). HL60 and THP-1 cells exhibiting the high LINC00504 expression were used for subsequent experiments. Taken together, aberrant high expression of LINC00504 may be involved in the regulation of AML progression.

LINC00504 knockdown suppressed AML cell proliferation and glycolysis, while inducing cell apoptosis. Then we examined the effects of LINC00504 on AML cell growth in *vitro*. Firstly, shRNA targeting LINC00504 (sh-LINC00504#1, #2, #3) was transfected into HL60 and THP-1 cells to decrease the expression of LINC00504. PCR results showed that the expression of LINC00504 was significantly downregulated in the sh-LINC00504 group (Figure 2A). The lowest knockdown efficiency was expressed in the sh-LINC00504#3 group. Therefore, sh-LINC00504#3 was selected as the most suitable knockdown plasmid. Cell viability was detected using CCK-8 and BrdU proliferation assays. CCK-8 results showed that LINC00504 knockdown decreased the viability of HL60 and THP-1 cells (Figure 2B). Analogously, the BrdU assay showed that LINC00504 silencing reduced the proportion of BrdU-positive cells (Figure 2C, Supplementary Figure S1A). Moreover, flow cytometry analysis demonstrated that LINC00504 knockdown exacerbated AML cell apoptosis (Figure 2D, Supplementary Figure S1B). ELISA analysis revealed that LINC00504 knockdown reduced glucose consumption, lactate production, and ATP level, suggesting that LINC00504 downregulation effectively restrained glycolysis (Figure 2E). Finally, western blotting was conducted to examine the expressions of Ki-67, cleaved caspase-3, HK2, and p53. Results showed that LINC00504 knockdown significantly decreased the protein expression of Ki-67 and HK2, whereas increased the expression of cleaved caspase-3 and p53 (Figures 2F, 2G). These above results indicated that LINC00504 exerted a promoting role in the malignant progression of AML.

Table 1. Correlation between LINC00504 expression and the clinicopathological features of 66 AML patients.

Characteristic	All cases	LINC00504 expression		p-value
		High (n=33)	Low (n=33)	
Age (years)				0.438
<55	43	23	20	
≥55	23	10	13	
Gender				0.467
Male	29	16	13	
Female	37	17	20	
WBC/ $\times 10^9$/l				0.027*
<10	18	5	13	
≥10	48	28	20	
FAB classification				0.171
M1	8	3	5	
M2	31	13	18	
M3	12	5	7	
M4	9	7	2	
M5	3	3	0	
M6	3	2	1	
Blast in BM				0.459
<50%	35	16	19	
≥50%	31	17	14	
Cytogenetics				0.028*
Favorable	20	8	12	
Intermediate	26	10	16	
Unfavorable	20	15	5	

Note: *p<0.05; Abbreviations: AML-acute myeloid leukemia; BM-bone marrow; FAB-French-American-British; WBC-white blood cell

LINC00504 mediated AML progression by interacting with MDM2. To further understand the promotion mechanism of LINC00504 in AML, we predicted the potential protein MDM2 that can bind to LINC00504 through a starBase database. Subsequently, pull-down and RIP assays were performed to validate the combination of LINC00504 and MDM2. Pull-down results showed that MDM2 was enriched in LINC00504-pulldown precipitates (Figure 3A). RIP analysis showed that LINC00504 directly interacted with MDM2 (Figure 3B). These results suggested that LINC00504 may play regulatory roles in AML through binding to MDM2. Subsequently, the expressions of LINC00504 in HL60 and THP-1 cells were increased by transfection of LINC00504 overexpression plasmid (Figure 3C). In expression analysis, LINC00504 knockdown downregulated MDM2 expression (Figure 3D). Without a doubt, overexpression of LINC00504 upregulated the MDM2 expression (Figure 3E). Furthermore, compared with healthy patients, MDM2 was highly expressed in AML patients (Figure 3F). Spearman correlation analysis showed that LINC00504 and MDM2 were positively correlated (Figure 3G). These findings suggested that LINC00504 could bind to MDM2 and positively regulate its expression.

LINC00504 facilitated AML cell growth by regulating MDM2. Based on these above results, we continued to examine the effects of LINC00504 on AML cell growth by interacting with MDM2. We first transfected sh-LINC00504 and MDM2 overexpression plasmids (MDM2) into HL60 and THP-1 cells to reduce LINC00504 expression and increase MDM2 expression, respectively. This result was confirmed by western blotting (Figure 4A). Results of cell function experiments *in vitro* showed that LINC00504 knockdown inhibited the proliferation and glycolysis of AML cells while promoting

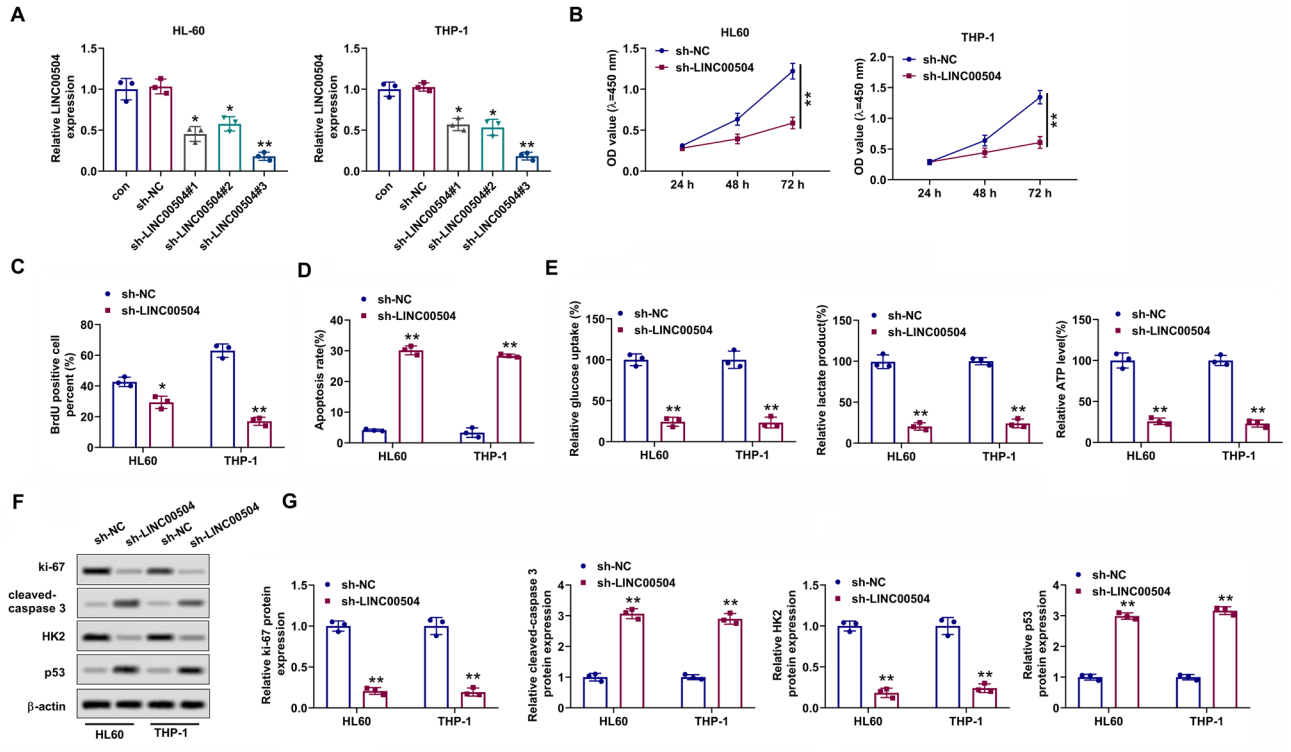

Figure 2. LINC00504 knockdown suppressed AML cell proliferation and glycolysis while inducing cell apoptosis. A) Efficiency of shRNA-mediated LINC00504 knockdown (sh-LINC00504#1, #2, #3) was determined using RT-PCR. *p<0.05 and **p<0.01 vs. sh-NC by Student's t-test. B) Cell viability of HL60 and THP-1 cells transfected with sh-NC or sh-LINC00504 was determined using CCK-8 assay. **p<0.01 vs. sh-NC by ANOVA. C) Cell proliferation of HL60 and THP-1 cells transfected with sh-NC or sh-LINC00504 was determined using BrdU staining. **p<0.01 vs. sh-NC by Student's t-test. D) Cell apoptosis of HL60 and THP-1 cells transfected with sh-NC or sh-LINC00504 was determined using flow cytometry. **p<0.01 vs. sh-NC by Student's t-test. E) Glycolytic phenotypes (glucose consumption, lactate production, and ATP level) of HL60 and THP-1 cells transfected with sh-NC or sh-LINC00504 were determined using ELISA assay. **p<0.01 vs. sh-NC by Student's t-test. F) Western Blot strip chart for different proteins (Ki-67, HK2, cleaved caspase-3, and p53). **p<0.01 vs. sh-NC by Student's t-test. G) The protein expression of Ki-67, HK2, cleaved caspase-3, and p53 of HL60 and THP-1 cells transfected with sh-NC or sh-LINC00504 was determined using western blotting. **p<0.01 vs. sh-NC by Student's t-test. The experiments are shown as means ± SD and experiments were performed at least in duplicate.

apoptosis. However, these above changes could be reversed by MDM2 overexpression (Figures 4B–4E, Supplementary Figures S2A, S2B). Western blotting showed that the inhibitory effect of LINC00504 downregulation on the expression of Ki-67 and HK2 and the promotion of cleaved caspase-3 and p53 expression in AML cells were alleviated by MDM2 overexpression (Figures 4F, 4G). Taken together, LINC00504 aggravated the progression of AML by upregulating MDM2.

LINC00504 knockdown reduced AML cell growth *in vivo*. Finally, we assessed the effect of LINC00504 on AML cell growth using a xenograft mouse model *in vivo*. Compared with the sh-NC group, the tumor size in the sh-LINC00504 group was significantly smaller (Figure 5A). Moreover, the tumor volume and weight in mice inoculated with sh-LINC00504 cells gradually became smaller with increasing time (Figures 5B, 5C). Meanwhile, the tumor weight in the sh-LINC00504 group was significantly lower than that in the sh-NC group (Figure 5D). Immunohistochemical results revealed that LINC00504 knockdown promoted the expression of cleaved caspase-3 and p53, and reduced the expression of MDM2, Ki-67, and HK2 (Figure 5E). Overall, these above findings indicated that LINC00504 exerts promoting effect during AML cell growth *in vivo* and *in vitro*.

Discussion

As a malignant tumor originating from hematopoietic stem cells (HSC), the main treatments of AML include but are not limited to chemotherapy, supportive therapy, and HSC transplantation. However, the median survival for AML patients, greater than 60 years, due to chemotherapy intolerance is 5–10 months, while the 5-year survival rate of AML patients under the age of 60 years is less than 40% [12, 13]. It is particularly urgent to find molecular biomarkers to target AML. In recent years, amounts of lncRNAs have been confirmed to be involved in the regulation of tumor cell functions and gene expression, especially in malignant blood disorders including leukemia [14]. In the present study, our data demonstrated

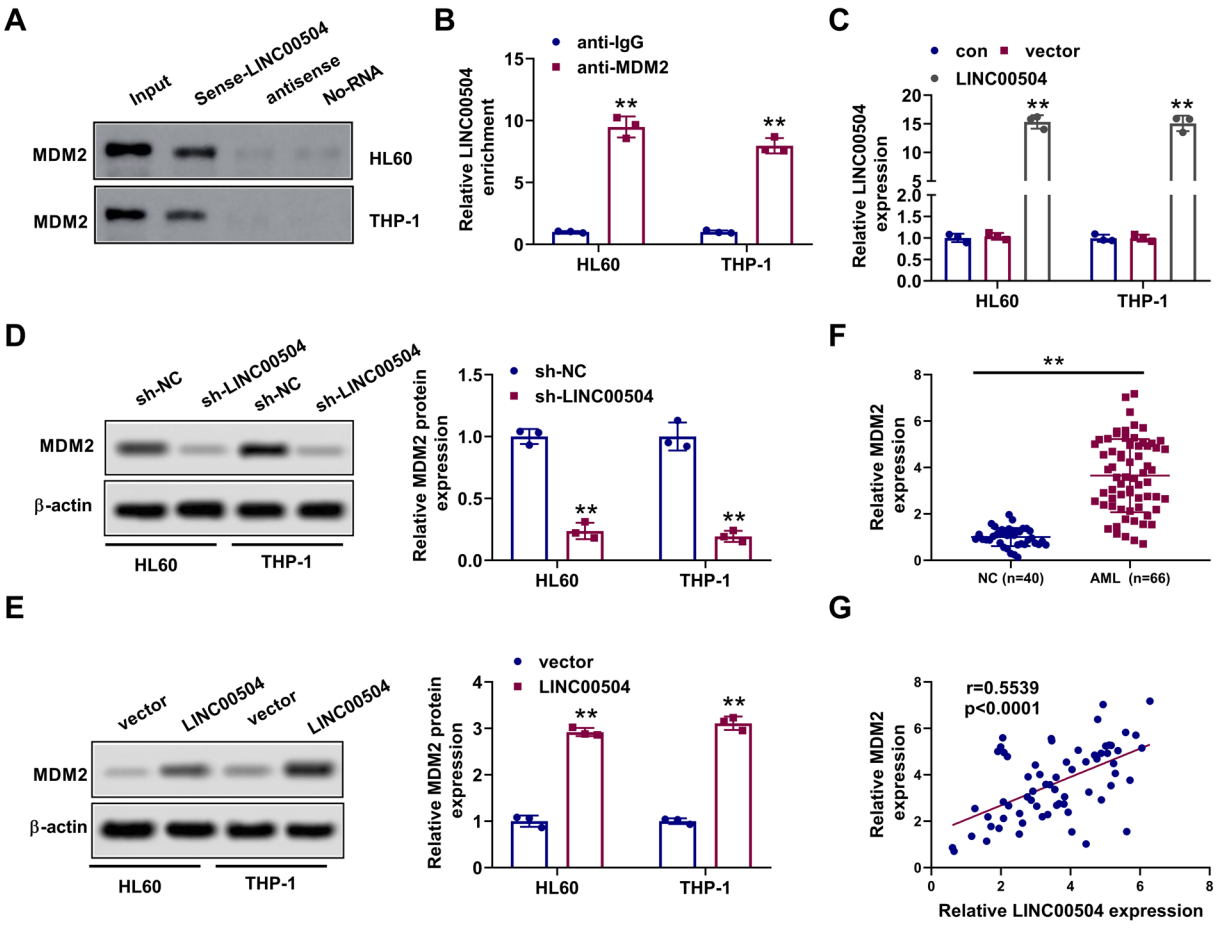

Figure 3. LINC00504 mediated AML progression by interacting with MDM2. A) Binding situation between LINC00504 and MDM2 was validated using a pull-down assay. B) The relationship between LINC00504 and MDM2 was validated using RIP assay. **p<0.01 vs. anti-IgG by Student's t-test. C) Efficiency of pcDNA-mediated LINC00504 overexpression was measured using RT-PCR. **p<0.01 vs. vector by Student's t-test. D) Effect of LINC00504 knockdown on MDM2 expression using western blotting. **p<0.01 vs. sh-NC by Student's t-test. E) Effect of LINC00504 overexpression on MDM2 expression using western blotting. **p<0.01 vs. vector by Student's t-test. F) MDM2 expression in AML clinical samples (n=40) and normal samples (n=66) was examined using RT-PCR. **p<0.01 vs. NC by Student's t-test. G) Correlation between LINC00504 and MDM2 was assessed using Spearman analysis. The experiments are shown as means ± SD and experiments were performed at least in duplicate.

that abnormally high expression of LINC00504 in AML clinical samples and cells is closely related to poor prognosis. Additionally, LINC00504 could aggravate the disease progression in AML via binding to MDM2.

As a novel lncRNA, LINC00504 has been proven to play an important regulatory role in many diseases. In the most frequently studied domain, oncology, LINC00504 as a cancer-promoting factor aggravated the majority of tumor progressions. For instance, LINC00504 promoted breast cancer cell growth and metastasis through modulating the miR-876-3p/HMGB3 axis [15]. Moreover, LINC00504 promoted glycolysis of ovarian cancer cells via downregulating miR-1244 expression [16]. In leukemia, a gene expression analysis of AML patients from TCGA database revealed that LINC00504 was highly expressed in AML patients, and high expression of LINC00504 was positively correlated with poorer survival outcomes in AML patients [9]. However, there is currently no research on LINC00504 to report the specific role and mechanisms of LINC00504 in AML. In our study, we found that LINC00504 expression was significantly increased in AML, and the reduction of LINC00504 effectively alleviated the AML cell growth and induced cell apoptosis. These inhibitory effects of LINC00504 knockdown were also confirmed by results *in vivo*. Otherwise, glycolysis metabolism, as an important manifestation of malignant phenotype aggravation in the tumor, is also gradually described in AML [17, 18]. Zhang et al. have confirmed that lncRNA UCA1 facilitated glycolysis by regulating miR-125a/HK2 and thus provoked chemoresistance in patients with childhood AML [19]. In this study, LINC00504 knockdown could inhibit the glycolysis process of AML cells by downregulating glycolysis-associated proteins (HK2) and controlling the glycolytic

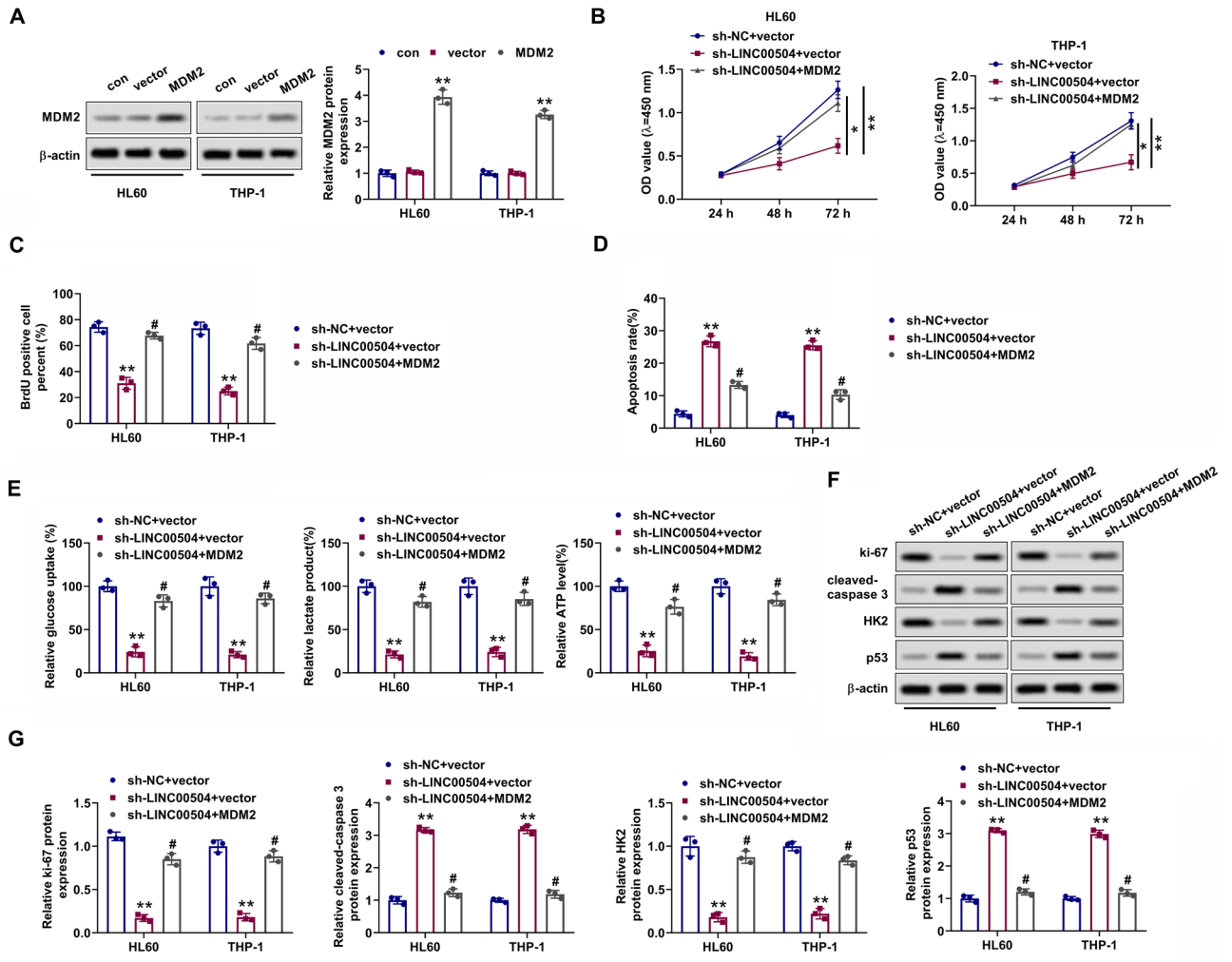

Figure 4. LINC00504 facilitated AML cell growth by regulating MDM2. A) The efficiency of pcDNA-mediated MDM2 overexpression was determined using RT-PCR. **p<0.01 vs. vector by Student's t-test. B) Cell viability of HL60 and THP-1 cells co-transfected with sh-LINC00504 and/or MDM2 was determined using CCK-8 assay. *p<0.05 and **p<0.01 vs. sh-LINC00504+vector by ANOVA. C) Cell proliferation of HL60 and THP-1 cells co-transfected with sh-LINC00504 and/or MDM2 was determined using BrdU staining. **p<0.01 vs. sh-NC+vector; #p<0.05 vs. sh-LINC00504+vector by Student's t-test. D) Cell apoptosis of HL60 and THP-1 cells co-transfected with sh-LINC00504 and/or MDM2 was determined using flow cytometry. **p<0.01 vs. sh-NC+vector; #p<0.05 vs. sh-LINC00504+vector by Student's t-test. E) Glycolytic phenotypes (glucose consumption, lactate production, and ATP level) of HL60 and THP-1 cells co-transfected with sh-LINC00504 and/or MDM2 were determined using ELISA assay. **p<0.01 vs. sh-NC+vector; #p<0.05 vs. sh-LINC00504+vector by Student's t-test. F) Western Blot strip chart for different proteins (Ki-67, HK2, cleaved caspase-3, and p53). G) The protein expression of Ki-67, HK2, cleaved caspase-3, and p53 of HL60 and THP-1 cells co-transfected with sh-LINC00504 and/or MDM2 was determined using western blotting. **p<0.01 vs. sh-NC+vector; #p<0.05 vs. sh-LINC00504+vector by Student's t-test. The experiments are shown as means ± SD and experiments were performed at least in duplicate.

metabolites (glucose consumption, lactate production, and ATP level). Overall, the malignant phenotypes of AML cells were exacerbated by LINC00504.

Next, we deeply explore the potential mechanisms of LINC00504 regulating AML. Different from the lncRNA/miRNA/mRNA regulatory network, lncRNAs could exert a crucial role in AML by binding to proteins. For example, Shi et al. reported that lncRNA SNHG16 inhibited the growth and metastasis of AML cells by binding to CELF2 to reduce the mRNA expression of CELF2 [20]. Zhang et al. manifested that LINC00319 interacting with RNA-binding protein (FUS) promoted AML cell growth and survival [21]. Here, we confirmed that LINC00504 could directly bind to MDM2 through pull-down and RIP analyses. MDM2 has been shown to be a molecular target for AML therapy by numerous studies. It is well known that the inactivation of wild-type p53 occurred in almost all AML subtypes, and MDM2, as a negative regulator of p53, could prevent p53 from binding to its target DNA by binding to p53, resulting in the transcriptional arrest and aggravating AML progression

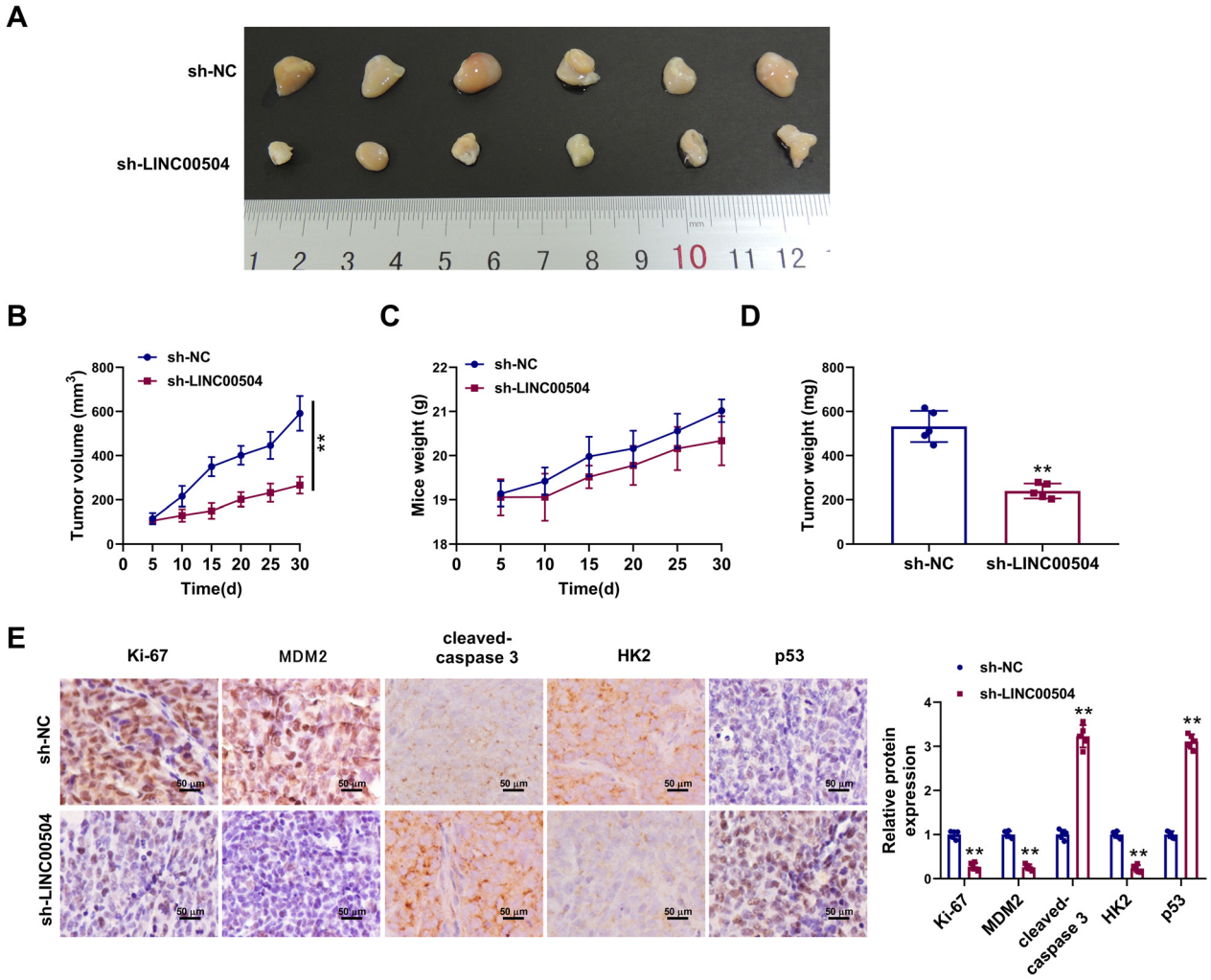

Figure 5. LINC00504 knockdown reduced AML cell growth *in vivo*. HL60 cells stably transfected with sh-LINC00504 (n=6) or sh-NC (n=6) were injected into mice to induce tumorigenesis. A) Representative images of tumor tissues from mice of each group 30 days post-inoculation. B) Tumor volume was measured every 5 d in each group. **p<0.01 vs. sh-NC by ANOVA. C) Mice weight was measured every 5 d in each group. D) Tumor weight was measured after AML cell growth for 30 d in each group. **p<0.01 vs. sh-NC by Student's t-test. E) Proteins (Ki-67, MDM2, cleaved caspase-3, HK2, and p53) of tumor tissues from mice in each group were analyzed using immunohistochemical staining. **p<0.01 vs. sh-NC by Student's t-test. The experiments are shown as means ± SD and experiments were performed at least in duplicate.

[22]. At present, given that the interaction of the MDM2-p53 pathway in AML, some drugs, for example RG7388 (MDM2 inhibitor), have shown good efficacy in phase I and II clinical studies, and RG7388 combined with cytarabine in a phase III study (NCT02545283) is undergoing evaluation [23]. In this study, LINC00504 directly bound to MDM2 and upregulated its expression. In addition, LINC00504 silencing effectively reduced the growth and glycolysis of AML cells and activated p53. These above changes could be reversed by MDM2 overexpression. In general, LINC00504 enhanced AML cell growth by interacting with RNA-binding protein (MDM2).

In conclusion, we confirmed the aberrant upregulation of LINC00504 in AML and for the first time characterized the function of LINC00504 in AML, showing that LINC00504 promoted AML cell growth and survival by binding to MDM2. Certainly, more detailed functions and downstream regulator mechanisms of LINC00504 in AML should be explored in future work. For *in vivo* studies, this study only detected the expression level of MDM2 by immunocytochemistry. We will continue to explore the regulation of MDM2 components by LINC00504 through a series of *in vivo* experiments, including overexpressing MDM2 in combination with sh-LIN00504.

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Supplementary information is available in the online version of the paper.

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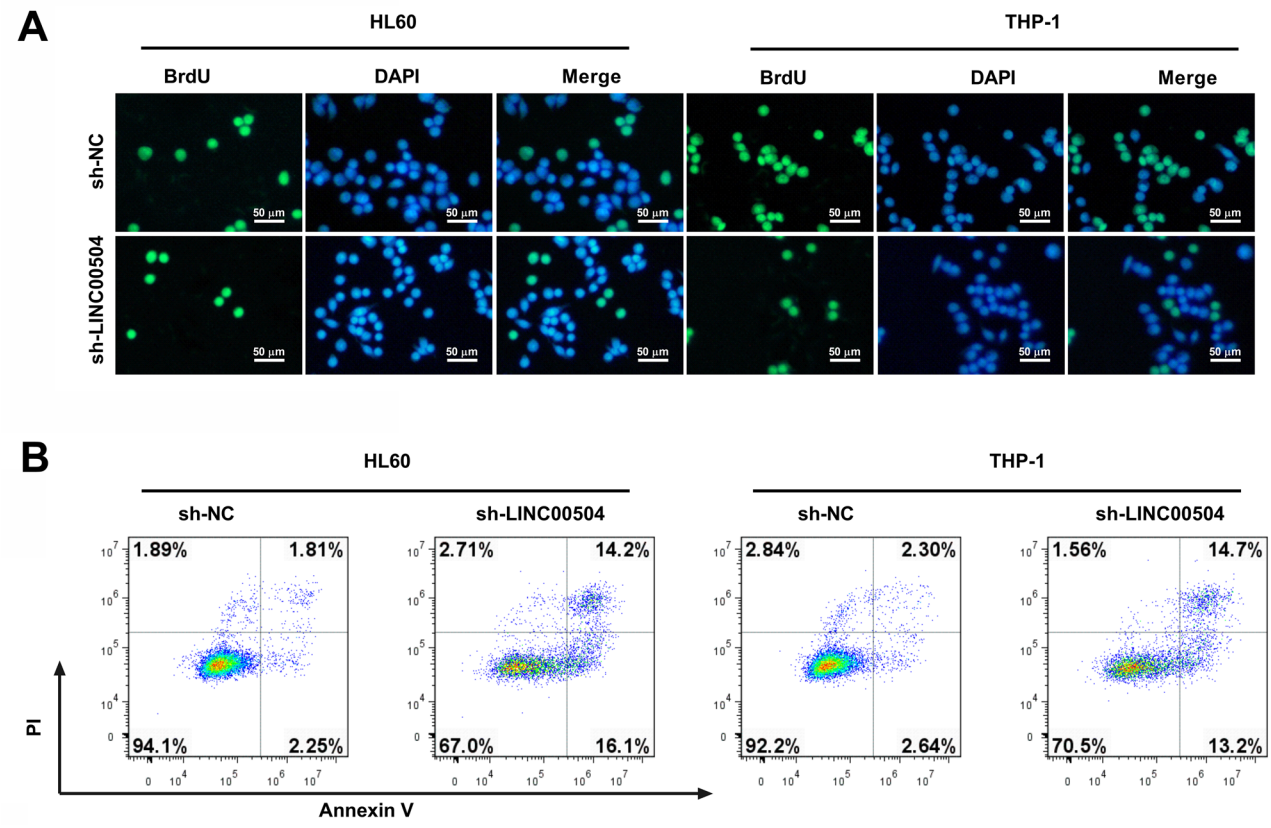
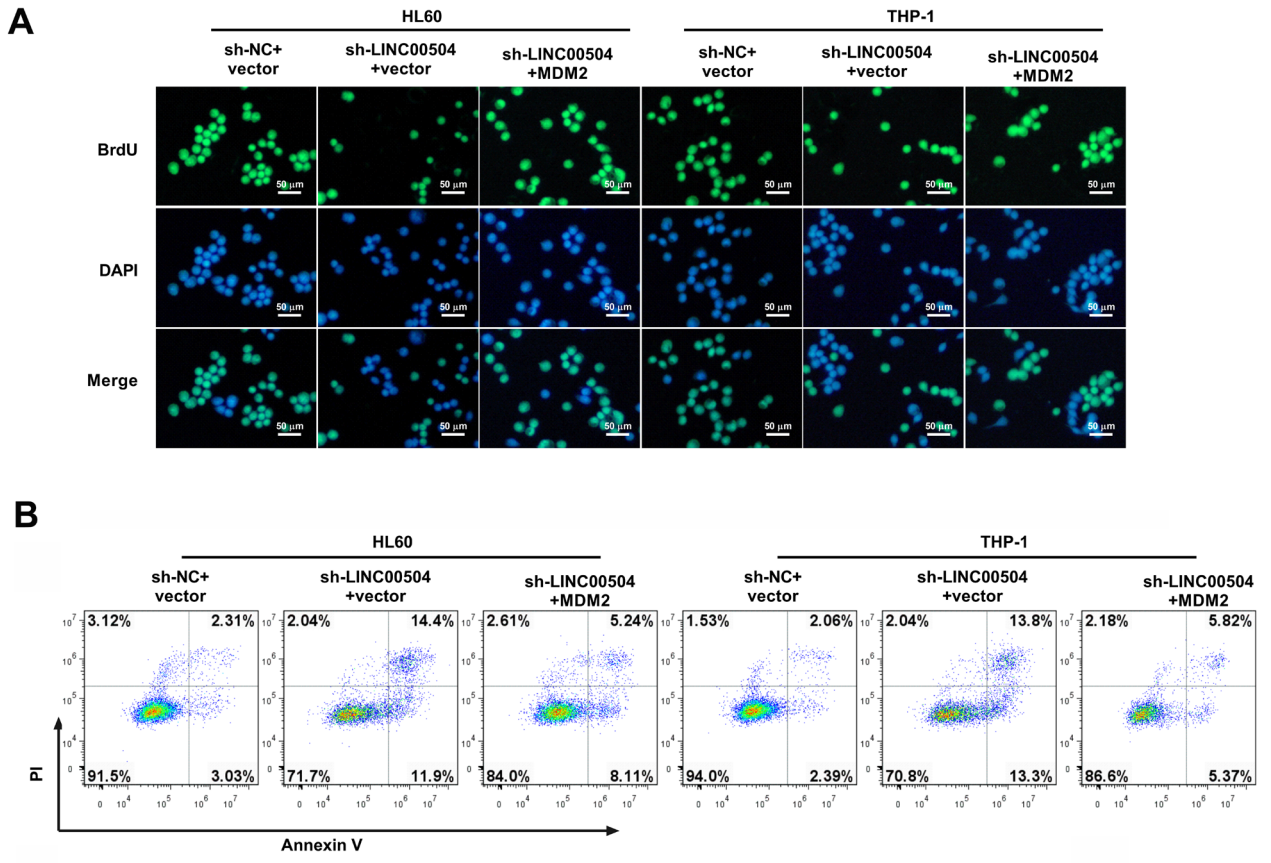
LINC00504 promotes the progression of acute myeloid leukemia by targeting MDM2

Jin-Hua YAN[1,*], Ling YAO[2], Ying TAN[3], Yue WANG[1]

Supplementary Information



Supplementary Figure S1. LINC00504 knockdown suppressed AML cell proliferation, but induced cell apoptosis. A) Cell proliferation of HL60 and THP-1 cells transfected with sh-NC or sh-LINC00504 was determined using BrdU staining. B) Cell apoptosis of HL60 and THP-1 cells transfected with sh-NC or sh-LINC00504 was determined using flow cytometry.



Supplementary Figure S2. MDM2 overexpression reversed the effect of LINC00504 knockdown on the proliferation and apoptosis of AML cells. A) Cell proliferation of HL60 and THP-1 cells co-transfected with sh-LINC00504 and/or MDM2 was determined using BrdU staining. B) Cell apoptosis of HL60 and THP-1 cells co-transfected with sh-LINC00504 and/or MDM2 was determined using flow cytometry.