Original Article

Circ_0012152 Accelerates Acute Myeloid Leukemia Progression through the miR-652-3p/*SOX4* Axis*

Ying CHEN[1, 2†], Bi-xia LI[2, 3†], Ting-ting NIU[1, 2†], Shu-jun YANG[2, 3], Li-chao WU[4], Le-huai SHI[2], Duo-bing ZOU[1, 2], Ning-ning WU[1, 2], Li-xia SHENG[2, 3], Xiao YAN[2, 3#], Gui-fang OUYANG[2, 3#], Qi-tian MU[1, 2, 5#]

[1]*Laboratory of Stem Cell Transplantation, The First Affiliated Hospital of Ningbo University, Ningbo, 315300, China*
[2]*Ningbo Clinical Research Center For Hematologic Malignancies, Ningbo, 315300, China*
[3]*Department of Hematology, The First Affiliated Hospital of Ningbo University, Ningbo, 315300, China*
[4]*School of Medicine, Hangzhou City University, Zhejiang University, Hangzhou, 310000, China*
[5]*Key Laboratory of Precision Medicine for Atherosclerotic Diseases of Zhejiang Province, Ningbo, 315300, China*

[Abstract] Objective: Acute myeloid leukemia (AML) is an aggressive hematological malignancy characterized by abnormal myeloid blast expansion. Recent studies have demonstrated that circular RNAs play a role in AML pathogenesis. In this study, we aimed to investigate the clinical significance of circ_0012152 in AML and elucidate its underlying molecular mechanism in the pathogenesis of this condition. **Methods:** Circ_0012152 expression was detected by quantitative real-time polymerase chain reaction in samples obtained from 247 patients with AML and 40 healthy controls. A systematic analysis of clinical characteristics and prognostic factors was also conducted. Cell growth was assessed using the Cell Counting Kit-8 (CCK-8) assay, and apoptosis and cell cycle progression were evaluated by flow cytometry. Moreover, RNA pull-down was performed to identify target microRNAs, and transcriptome RNA sequencing and bioinformatics analyses were utilized to identify downstream mRNA targets. **Results:** Circ_0012152 was significantly upregulated in samples from patients with AML and served as an independent adverse prognostic factor for overall survival (OS) (hazard ratio: 2.357; 95% confidence interval 1.258–4.415). The circ_0012152 knockdown reduced cell growth, increased apoptosis, and inhibited cell cycle progression in AML cell lines. RNA pull-down and sequencing identified miR-652-3p as a target microRNA of circ_0012152. Cell growth inhibition by circ_0012152 knockdown was significantly relieved by miR-652-3p inhibitors. We suggested that miR-652-3p targeted *SOX4*, as the decrease in *SOX4* expression resulting from circ_0012152 knockdown was upregulated by miR-652-3p inhibitors in AML cells. **Conclusion:** Circ_0012152 is an independent poor prognostic factor for OS in AML, and it promotes AML cell growth by upregulating *SOX4* through miR-652-3p.

Keywords: acute myeloid leukemia; circ_0012152; miR-652-3p; *SOX4*; cell growth

DOI https://doi.org/10.1007/s11596-024-2878-y

Acute myeloid leukemia (AML) is a malignancy of the hematopoietic system that is characterized by significant clinical heterogeneity and diverse prognoses[1]. A range of therapies, including chemotherapeutics, autologous/allogeneic hematopoietic stem cell transplantation, and chimeric antigen receptor T-cell therapy, have demonstrated success in treating AML, but the continued presence of poor outcomes for patients necessitates the development of more effective treatment approaches[2–4].

Advances in the field of epigenetics have increased the attention given to circular RNAs (circRNAs) as potential prognostic biomarkers and therapeutic targets for AML[5, 6]. CircRNAs, which were once considered intermediates or byproducts produced during splicing, have been identified in numerous mammals[7]. CircRNAs have emerged as potential biomarkers for various diseases, particularly malignancies[8]. Recent studies have demonstrated associations between circRNAs and metastasis, drug resistance, tumor stage, and overall survival (OS) in colorectal, gastric, lung, bladder, and breast cancer, hepatocellular carcinoma, leukemia, and other types of cancer[9–11]. Moreover, emerging evidence highlights the significant role of circRNAs in the development of AML. For example, the knockdown of circ-ANXA2 suppresses growth, enhances apoptosis and increases the chemosensitivity of THP-1 and KG-1 cells[12]. Additionally, circ_0079480 enhances AML invasion through the miR-654-3p/HDGF axis[13]. Fusion-circRNAs have also been implicated in the inhibition of cell apoptosis and in AML progression[14].

The prevailing view is that circRNAs competitively bind to specific microRNAs (miRNAs) and interact with RNA-binding proteins to modulate tumor progression[15, 16]. MiRNAs, which consist of 21–25 nucleotides, regulate

Ying CHEN, E-mail: chenyingivy123@163.com; Bi-xia LI, E-mail: libixia421@qq.com; Ting-ting NIU, E-mail: 161075776@nbu.edu.cn
†The authors contributed equally to this work.
#Corresponding authors, Qi-tian MU, E-mail: muqitian@163.com; Gui-fang OUYANG, E-mail: nbhematology@163.com; Xiao YAN, E-mail: yanxiao.nb@outlook.com
*This research was supported by grants from the Natural Science Foundation of Zhejiang Province (No. LY20H080001), and Medical and Health Science and Technology Projects of Zhejiang Province (No. 2021KY997, No. 2022KY306, No. 2022KY316, No. 2023KY263).
Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11596-024-2878-y) contains supplementary material, which is available to authorized users.

gene expression after transcription or by degrading target gene messenger RNA (mRNA)[17, 18]. Therefore, the identification of reliable circRNAs, miRNAs, and downstream mRNAs is crucial for understanding the pathogenesis of AML. A previous study[19] proposed that hsa_circ_0012152 serves as a promising diagnostic biomarker for AML and that it may be a useful classification biomarker for differentiating AML from acute lymphoblastic leukemia (ALL).

Bioinformatics analysis suggested that hsa_circ_0012152 might be involved in the initiation and development of AML through the miR-491-5p/EGFR/MAPK1 axis or the miR-512-3p/EGFR/MAPK1 axis[19]. Moreover, other studies have reported that hsa_circ_0012152 plays a critical role in AML via the miR-625-5p/SOX12 axis, the miR-30a/MYSM1 and IER2 axes, and the miR-330-5p/*SOX4* axis[20–22].

In this study, we systematically analyzed the association between circ_0012152 and AML, and a novel target miRNA of circ_0012152 was identified, which helped to further elucidate the pathological mechanism underlying AML.

1 METHODS AND MATERIALS

1.1 Patients and Samples

Bone marrow mononuclear cells were obtained from 298 individuals, including 26 patients with acute promyelocytic leukemia (APL), 221 with non-APL AML, 11 patients with complete remission after chemotherapy, and 40 healthy volunteers. These samples were stored at The First Affiliated Hospital of Ningbo University. The karyotypes of all patients were determined according to the 2017 European LeukemiaNet classification of AML[23].

The clinical characteristics of the patients with AML are presented in table 1. The study was approved by the Ethics Committee of the First Affiliated Hospital of Ningbo University (Ningbo First Hospital, China) and was conducted in compliance with relevant medical ethics regulations. Informed consents were obtained from all subjects or their legal guardians.

1.2 Cell Culture and Transfection

AML cell lines (MOLM-13, OCI-AML2, and NB4) and a human embryonic kidney cell line (HEK-293T) were obtained from the Zhejiang Provincial Key Laboratory of Hematopoietic Malignancy. The cells were cultured in Iscove's modified Dulbecco's medium or Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco) at 37°C in an incubator with 5% carbon dioxide. All cell lines were routinely tested for mycoplasma contamination.

Lentiviruses for circ_0012152 [short hairpin (sh)-circ_0012152), negative control (sh-NC), and several miRNA inhibitors were designed by GenePharma (China). Lentiviral transduction of cell lines was performed according to the manufacturer's protocol. Lipofectamine 3000 reagent (Thermo Fisher, USA) was used to transfect the miRNA inhibitors into the infected cells. The sequences of the lentivirus and miRNA inhibitor sequences are provided in table S1.

1.3 RNA Isolation and Real-time Quantitative Polymerase Chain Reaction

Total RNA was extracted from bone marrow samples or cells using RNAiso Plus (Takara, Japan). Complementary DNA (cDNA) was synthesized by reverse transcription of 1 μg of RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher) for amplification with TB Green Premix Ex Taq Ⅱ (Takara) in a real-time quantitative polymerase chain reaction (qRT-PCR) system. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method, and GAPDH was used as the internal standard. The primers used are listed in table S2.

1.4 RNase R assay

The RNase R reaction system was prepared according to the RNase R manual (Lucigen, USA). RNA (5 μg) was treated with the aforementioned system at 37°C for 30 min. RNase-free water was used as a control (Mock). Both groups were subjected to qRT-PCR. Circ_00012152 was identified by the RNase R assay (fig. 1A).

1.5 Cell Counting Kit-8 Assay

Infected cells were seeded into 96-well plates at a density of 5×10^4 cells/well and incubated for 0, 24, 48, 72 or 96 h. After incubation, 10 μL of Cell Counting Kit-8 solution (NCM Biotech, China) was added to each well and incubated for 2 h. Cell viability was measured using a microplate reader at an absorbance (*A*) of 450 nm.

1.6 Apoptosis Assay

Infected cells were washed with prechilled phosphate-

Table 1 The expression of circ_0012152 in relation to clinical and laboratory features in AML patients

Characteristics		hsa_circ_0012152		*P* value
		Low expression group	High expression group	
Age (years), median (range)		54.5 (18–92)	51 (17–84)	0.480
Sex	Male	44	55	0.227
	Female	33	51	
WBC ($\times10^9$ cells/L), median (range)		17.39 (0.01–260.2)	21.3 (0.2–229.9)	0.870
Hb (g/L), median (range)		56 (3.6–136)	64.5 (5–135)	0.340
PLT ($\times10^9$ cells/L), median (range)		62 (2.95–556)	44.5 (2–875)	0.650
Blast in bone marrow (%)		51.50 (24–94)	69.75 (21–94)	<0.001
Karyotype	Good	8	9	0.077
	Moderate	49	74	
	Poor	15	8	
FLT3-ITD mutation		15.79%	28.87%	0.043

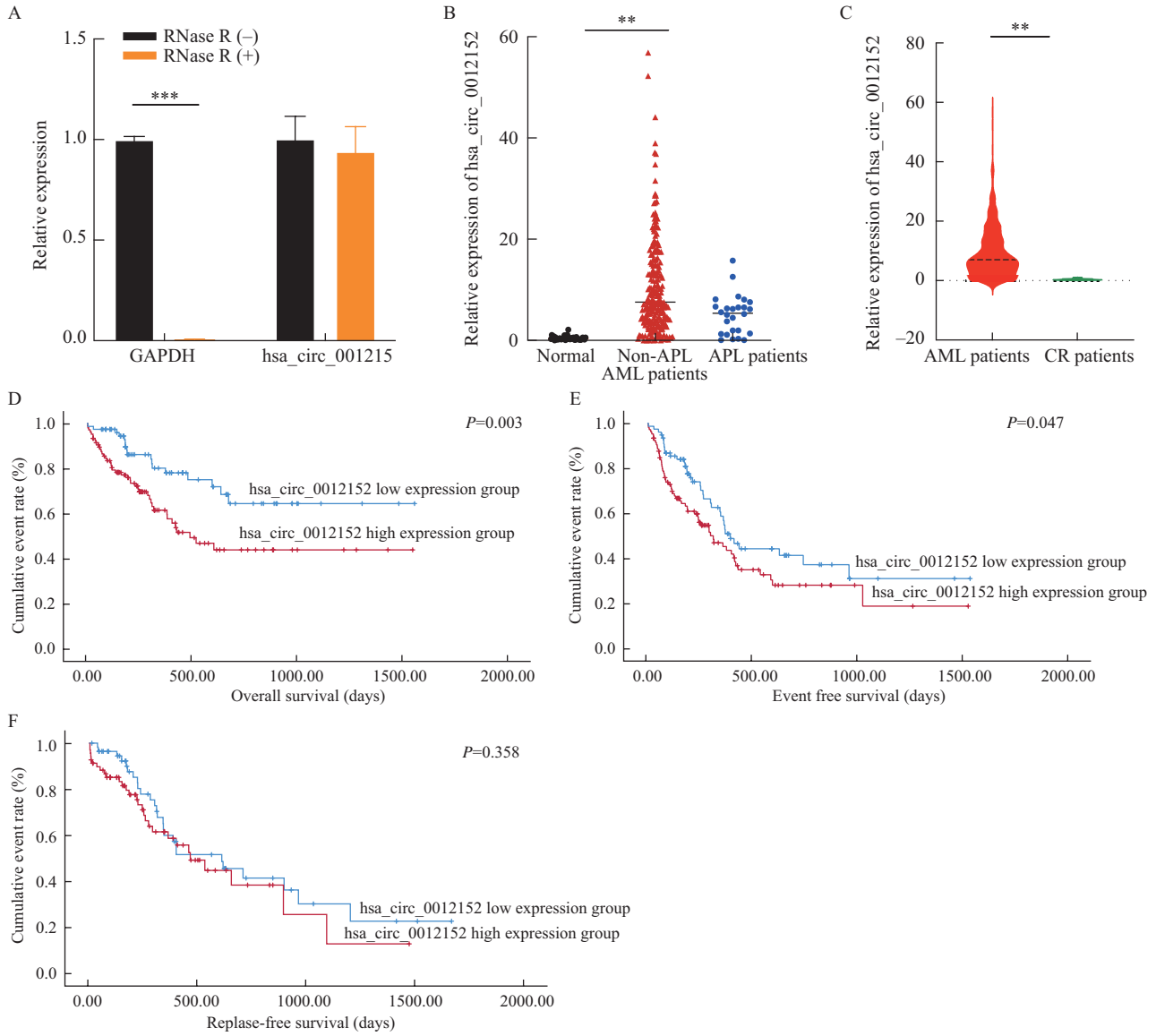


Fig. 1 Circ_0012152 is upregulated and an independent adverse factor in adult AML patients

A: Circ_0012152 was not degraded by RNase R, while GAPDH was degraded by RNase R; B: The expression levels of circ_0012152 in the non-APL AML, APL, and normal control groups were compared ($^{**}P<0.01$); C: Expression of circ_0012152 in patients who achieved primary AML and AML CR; D: comparison of OS between the circ_0012152 low-expression group and high-expression group in AML patients; E: comparison of EFS between the circ_0012152 low-expression group and high-expression group in AML patients; F: comparison of RFS between the circ_0012152 low-expression group and high-expression group in AML patients

buffered saline (PBS), resuspended in 1× binding buffer, and stained with Annexin V-PE (MultiSciences, China) for 30 min in the dark at room temperature according to the manufacturer's instructions. Subsequently, flow cytometry was used to analyze the number of apoptotic cells.

1.7 Cell Cycle Analysis

Infected cells were resuspended in PBS, and after centrifugation, DNA-staining solution (MultiSciences) was added to the tube and incubated for 30 min in the dark at room temperature. The cell cycle distribution was analyzed by flow cytometry.

1.8 Western Blotting

Infected MOLM-13 cells were lysed in radioimmunoprecipitation assay buffer supplemented with phenylmethanesulfonyl fluoride (Biyotime, China). After centrifugation at 12 000 r/min at 4°C, the supernatant was collected, and the protein concentration was determined using a bicinchoninic acid assay kit (Beyotime, China). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Solarbio, China) according to their molecular weight. The proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Nonspecific antibody binding sites were blocked with 5% skim milk powder (Solarbio). Primary rabbit anti-human antibodies were diluted in Tris-hydrochloride and Tween-20 and incubated with the PVDF membrane. The PVDF membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000, CST, USA). The protein bands were visualized using an enhanced chemiluminescence kit (Beyotime). The grayscale value of each target protein band, normalized to the β-actin intensity, was quantified using ImageJ software (version

1.52). Primary antibodies (all from Abcam, UK, at a dilution of 1:1000) against the following proteins were used: cleaved caspase-3, cyclin D4, cyclin D6, LC3, p62, and β-actin.

1.9 RNA Fluorescence *In Situ* Hybridization

To determine the intracellular localization of circ_0012152, RNA fluorescence *in situ* hybridization (FISH) was performed following the protocol of Geneseed Biotech (China). MOLM-13 cells were rinsed with PBS and fixed in 4% formaldehyde in RNase-free PBS for 5 min at room temperature. The samples were hybridized with the circ_0012152 probe overnight at 37°C in the dark. After hybridization, the slides were washed in 2× SSC for 5 min at room temperature. The nuclei were counterstained with DAPI (LBP, China) for 20 min in the dark. Images were acquired using a BX51 confocal microscope (Olympus, Japan).

1.10 RNA Pull-down Assay

An RNA pull-down assay (Geneseed Biotech) was used to identify miRNAs associated with circ_0012152. Circ_0012152-MS2 and MS2-CP vectors were cotransfected into 293T cells, and MS2-CP expression was induced. Subsequently, the MS2-CP-MS2-circRNA complex was formed and pulled down. Next-generation sequencing was used to detect the complex containing miRNAs bound to circ_0012152. The sequences of the miRNA inhibitors are listed in table S3.

1.11 Transcriptome RNA Sequencing Assay

RNA-seq was performed by the Beijing Genomics Institute (BGI, China) according to the following steps: (1) the cells were collected after lentivirus infection; (2) total RNA was extracted; (3) mRNA was enriched; (4) double-stranded cDNA was synthesized; and (5) the data were sequenced and analyzed.

1.12 Bioinformatics and Statistical Analysis

The $2^{-\Delta\Delta CT}$ method of relative quantification was used to analyze the circRNA expression of samples from healthy controls and patients with AML. The target miRNAs and genes of circ_0012152 were predicted using starBase (http://starbase.sysu.edu.cn). Statistical significance was determined using SPSS software (version 22.0; IBM Corporation, USA). Differences in the distribution of continuous variables between groups were identified using the Mann-Whitney *U* test or the *t* test, and differences between categorical variables were analyzed using the Chi-square test. Survival analyses were performed using the Kaplan-Meier method, with comparisons made using the log-rank test. Multivariate analyses were conducted using the Cox proportional hazards regression model. All the statistical tests were performed with 95% confidence intervals (CIs). $P<0.05$ was considered to indicate statistical significance.

2 RESULTS

2.1 Circ_00012152 Upregulation Indicates an Adverse Acute Myeloid Leukemia Prognosis

The expression of circ_0012152 was detected by qRT-PCR in samples from 40 healthy controls and 247 patients with primary AML. The circ_0012152 level was significantly higher in the samples from patients with AML than in those from healthy controls (median: 7.06 *vs*. 0.33; $P<0.001$). The circ_0012152 expression level was significantly higher in samples from patients with non-APL AML than in those from patients with APL (median: 7.56 *vs*. 5.39; $P=0.004$) (fig. 1B). The expression of circ_0012152 in samples from patients with complete remission (CR) of AML was lower than that in those from patients with newly diagnosed AML (median: 7.06 *vs*. 0.39; $P<0.001$) (fig. 1C).

R software was used to divide the 183 patients with non-APL AML into two groups according to the expression of circ_0012152. There were 106 patients in the low circ_0012152 expression group and 77 in the high circ_0012152 expression group. The relationships between clinical laboratory features and circ_0012152 expression were analyzed. The high circ_0012152 expression group had a greater proportion of immature bone marrow cells (median: 69.75% *vs*. 51.50%; $P<0.001$) and a greater percentage of FLT3-internal tandem duplication (ITD) mutations (28.87% *vs*. 15.79%; $P<0.05$) than the low circ_0012152 expression group. However, no significant differences in age, sex, white blood cell (WBC) count, hemoglobin (HB) level, platelet (PLT) count, karyotype, or 2022 World Health Organization subtype distribution were detected between the two groups (table 1).

The median follow-up time for the 183 patients with non-APL AML was 259 days. K-M analysis revealed that patients in the high circ_0012152 expression group had shorter overall survival (OS) (2-year OS rate: 44.0% *vs*. 64.5%; $P=0.003$) (fig. 1D) and shorter event-free survival (EFS) (median: 301 *vs*. 395 days; $P=0.049$, fig. 1E) than those in the low circ_0012152 expression group. However, there was no significant difference in relapse-free survival (RFS) (median: 466 days *vs*. 611 days; $P=0.358$, fig. 1F) between the two groups. Furthermore, age (>65 years) and karyotype (high risk) were identified as adverse prognostic factors for OS, while karyotype (high risk) was identified as an adverse prognostic factor, and *CEBPA* double mutation was identified as a favorable factor for EFS ($P<0.05$). Prognostic factors with $P<0.1$ were selected for multivariable analysis. High circ_0012152 expression and karyotype (high risk) were found to be independent adverse prognostic factors for OS (HR 2.357, 95% CI 1.258–4.415; HR 2.651, 95% CI 1.374–5.114, respectively), and karyotype (high risk) was found to be an independent adverse prognostic factor for EFS (HR 2.1499, 95% CI 1.331–3.469). Additionally, *CEBPA* double mutation was a favorable independent prognostic factor for EFS (HR 0.321, 95% CI 0.130–0.793) (table 2).

2.2 Circ_0012152 Knockdown Inhibits AML Cell Growth

To investigate the impact of circ_0012152 on AML cells, a lentiviral vector carrying an shRNA specific for circ_0012152 was used to infect the MOLM-13, OCI-AML2, and NB4 cell lines. The knockdown efficiency was assessed by qRT-PCR (fig. 2A). CCK-8, flow cytometry, and Western blotting assays indicated that circ_0012152

Table 2 Univariate and multivariate Cox regression analysis of prognostic factors in patients with AML

Factors	Overall survival				Event-free survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
hsa_circ_0012152 high expression	2.292 (1.302–4.036)	0.004	2.357 (1.258–4.415)	0.007	1.517 (1.003–2.296)	0.049	1.411 (0.886–2.248)	0.147
Age (>65 years)	1.729 (1.034–2.890)	0.037	1.592 (0.865–2.930)	0.135	1.226 (0.810–1.854)	0.336	–	–
Sex (male)	1.595 (0.940–2.707)	0.083	0.535 (0.286–1.004)	0.051	0.809 (0.539–1.213)	0.305	–	–
WBC (≥30×10^9/L)	1.609 (0.963–2.687)	0.069	1.557 (0.843–2.877)	0.158	1.312 (0.877–1.962)	0.186	–	–
Karyotype (high-risk)	2.092 (1.172–3.733)	0.012	2.651 (1.374–5.114)	0.004	2.081 (1.299–3.335)	0.002	2.1499 (1.331–3.469)	0.002
NPM1 mutation	1.447 (0.829–2.525)	0.193	–	–	1.421 (0.922–2.191)	0.111	–	–
FLT3-ITD mutation	1.747 (0.955–3.196)	0.070	1.779 (0.870–3.639)	0.114	1.598 (0.981–2.603)	0.060	1.529 (0.870–2.688)	0.140
IDH1 mutation	0.785 (0.279–2.205)	0.646	–	–	0.717 (0.327–1.572)	0.406	–	–
IDH2 mutation	1.577 (0.780–3.188)	0.205	–	–	1.063 (0.596–1.895)	0.836	–	–
DNMT3a mutation	1.549 (0.807–2.971)	0.188	–	–	1.103 (0.638–1.907)	0.725	–	–
P53 mutation	1.636 (0.558–4.793)	0.370	–	–	1.686 (0.694–4.095)	0.248	–	–
TET2 mutation	0.987 (0.366–2.660)	0.980	–	–	0.740 (0.305–1.795)	0.506	–	–
CEBPA double mutation	0.242 (0.059–0.997)	0.050	0.378 (0.09–1.589)	0.184	0.321 (0.130–0.793)	0.014	0.326 (0.117–0.902)	0.031

knockdown significantly inhibited cell growth (fig. 2 B), induced apoptosis (fig. 2C), and arrested the cell cycle at the G0/G1 phase (fig. 2E) in MOLM-13 and OCI-AML2 cells. Correspondingly, increased cleaved caspase-3 protein (fig. 2D) and decreased CDK4 and CDK6 proteins (fig. 2F) were observed in the knockdown groups.

2.3 Inhibition of miRNA-652-3p Alleviates Cell Growth Inhibition Caused by Circ_0012152 Knockdown

To investigate the subcellular localization of circ_0012152, a Cy3-labeled circ_0012152 probe for RNA-FISH was synthesized. RNA-FISH demonstrated that circ_0012152 was predominantly enriched in the cytoplasm of MOLM-13 cells. Subsequently, circ_0012152 was overexpressed in 293T cells. The circRNA compound was pulled down, and the enrichment fold change in circ_0012152 was determined by qRT-PCR. Notably, the circRNA enrichment group exhibited significantly higher levels of circ_0012152 expression than the negative control group (fig. 3A). Next-generation sequencing revealed that 133 miRNAs were significantly upregulated in the circRNA enrichment group (fig. 3B).

A total of 6 miRNAs with the lowest *P* values in the log2fold change ≥2 cohort in the pull-down assay, namely, hsa-miR-20a-5p, hsa-miR-652-3p, hsa-miR-455-5p, hsa-miR-1246, hsa-miR-98-5p, and hsa-miR-590-3p, were selected. Inhibitors of these miRNAs were synthesized and transfected into MOLM-13 cells with circ_0012152 knockdown. The preliminary screening results obtained using the CCK-8 assay indicated that upon exposure to miR-590-3p and miR-652-3p inhibitors, the cell growth rate was significantly higher than that of the NC group (*P*=0.001 and *P*<0.001, respectively) (fig. 3C). However, in the NC-circ-0012152 group, the growth of MOLM-13 cells transfected with the miR-590-3p inhibitor was significantly increased (*P*=0.003), while the miR-652-3p inhibitor had no discernible effect on cell growth (*P*=0.293, fig. 3D), which indicated that miR-652-3p may be a target miRNA of circ-0012152.

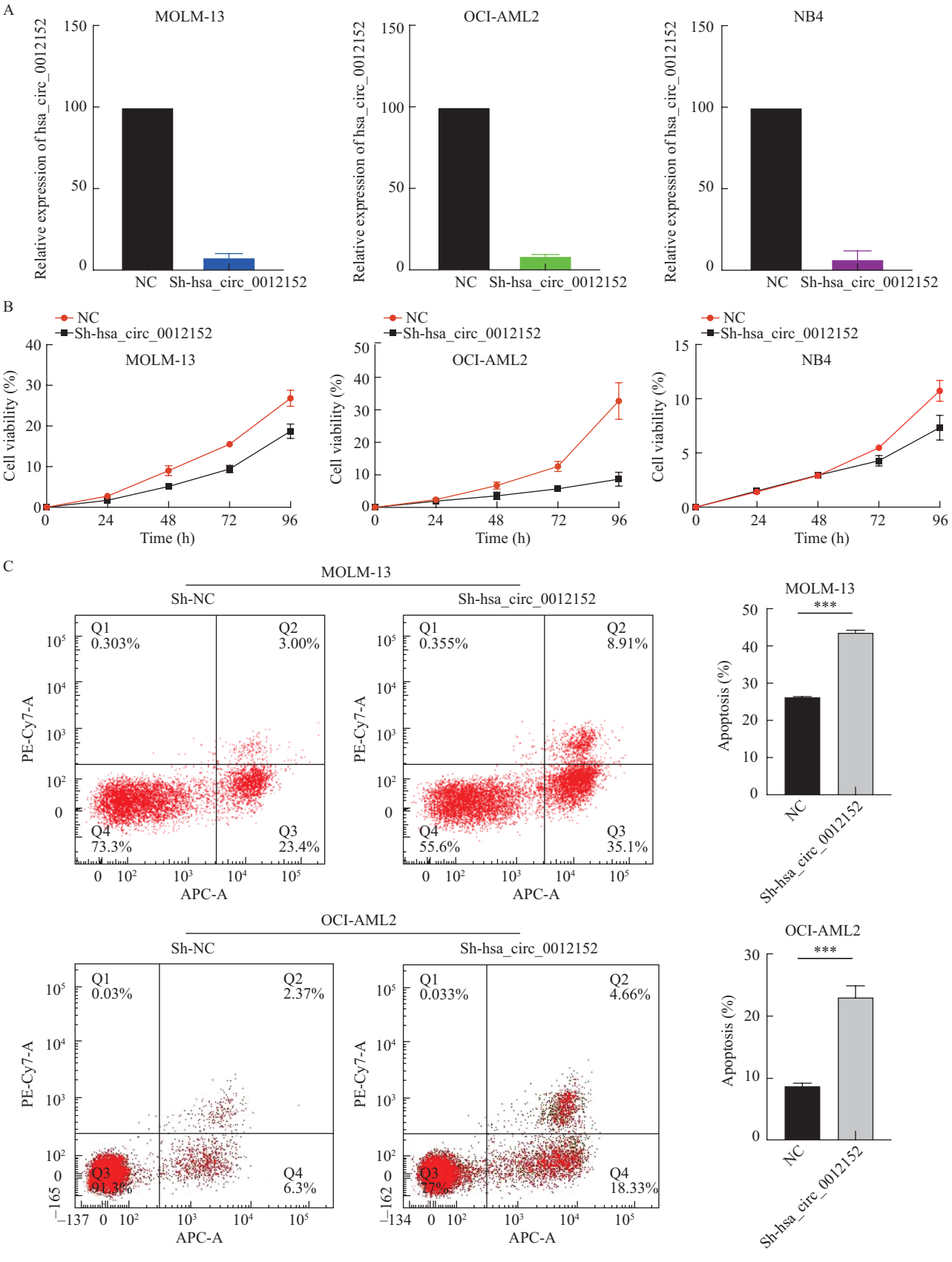
Next, the miR-652-3p inhibitor was transfected into MOLM-13 cells, which relieved the growth inhibition (fig. 3E). Bioinformatics analysis suggested that circ_0012152 had binding sites on miR-652-3p (fig. 3F). These findings support the notion that circ_0012152 may function as a miR-652-3p sponge in AML cells.

2.4 Circ_0012152 Regulates *SOX4* Expression through miR-652-3p

Transcriptome RNA sequencing was performed in OCI-AML2 cells with circ_0012152 knockdown to explore potential regulatory pathways. A total of 1427 differentially expressed genes (DEGs) were identified in the circ_0012152 knockdown group, including 868 upregulated genes and 559 downregulated genes (fig. 4A). All DEGs were subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses. The results of the GO analysis of the upregulated DEGs (fig. 4B) showed that these genes might play a role in biological processes such as “secretory granule lumen”, “cytoplasmic vesicle lumen”, and “vesicle lumen”, while the downregulated DEGs (fig. 4C) were involved in “regulation of membrane potential”, “Wnt signaling pathway”, and “cell-cell signaling by Wnt”, among others. KEGG analysis (fig. 4D) revealed that the DEGs might participate in pathways such as “Pathways in cancer”, “Phagosome pathway”, “PI3K-Akt signaling pathway”, “NOD-like receptor signaling pathway”, and “hematopoietic cell lineage”, among others.

By combining the predicted target genes of miR-652-3p from the Starbase database with the downregulated genes identified through transcriptome RNA sequencing, 3 genes were identified: *PLXND1*, *SOX4* and *DAAM1* (fig. 4E). Among these genes, *SOX4* was the most significantly downregulated gene, as confirmed by qRT-PCR. The results demonstrated that, compared to that in the NC circ_0012152 group, the relative expression of *SOX4* in the circ_0012152 knockdown group was significantly reduced (fig. 4F and 4G). Additionally,

SOX4 expression was also examined in the circ_0012152-knockdown MOLM-13 cell line transfected with miR-652-3p inhibitors. The miR-652-3p inhibitor partially restored *SOX4* expression, which was downregulated by circ_0012152 knockdown in MOLM-13 cells ($P<0.001$, fig. 4H). Thus, it was hypothesized that circ_0012152 might function as a sponge for miR-652-3p, subsequently promoting SOX4 expression (fig. 5).



(Continued to the next page)

(Continued from the previous page)

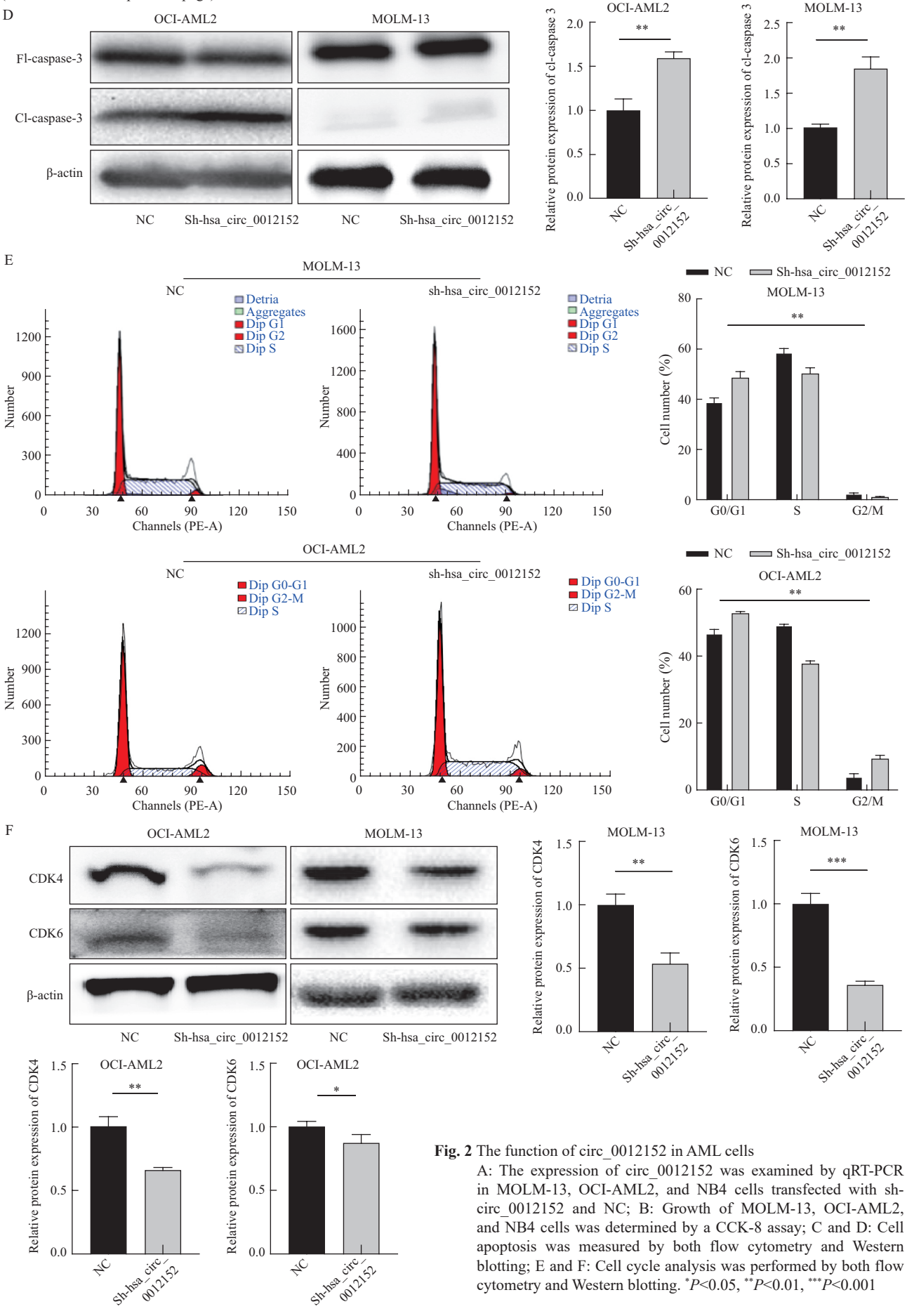


Fig. 2 The function of circ_0012152 in AML cells

A: The expression of circ_0012152 was examined by qRT-PCR in MOLM-13, OCI-AML2, and NB4 cells transfected with sh-circ_0012152 and NC; B: Growth of MOLM-13, OCI-AML2, and NB4 cells was determined by a CCK-8 assay; C and D: Cell apoptosis was measured by both flow cytometry and Western blotting; E and F: Cell cycle analysis was performed by both flow cytometry and Western blotting. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

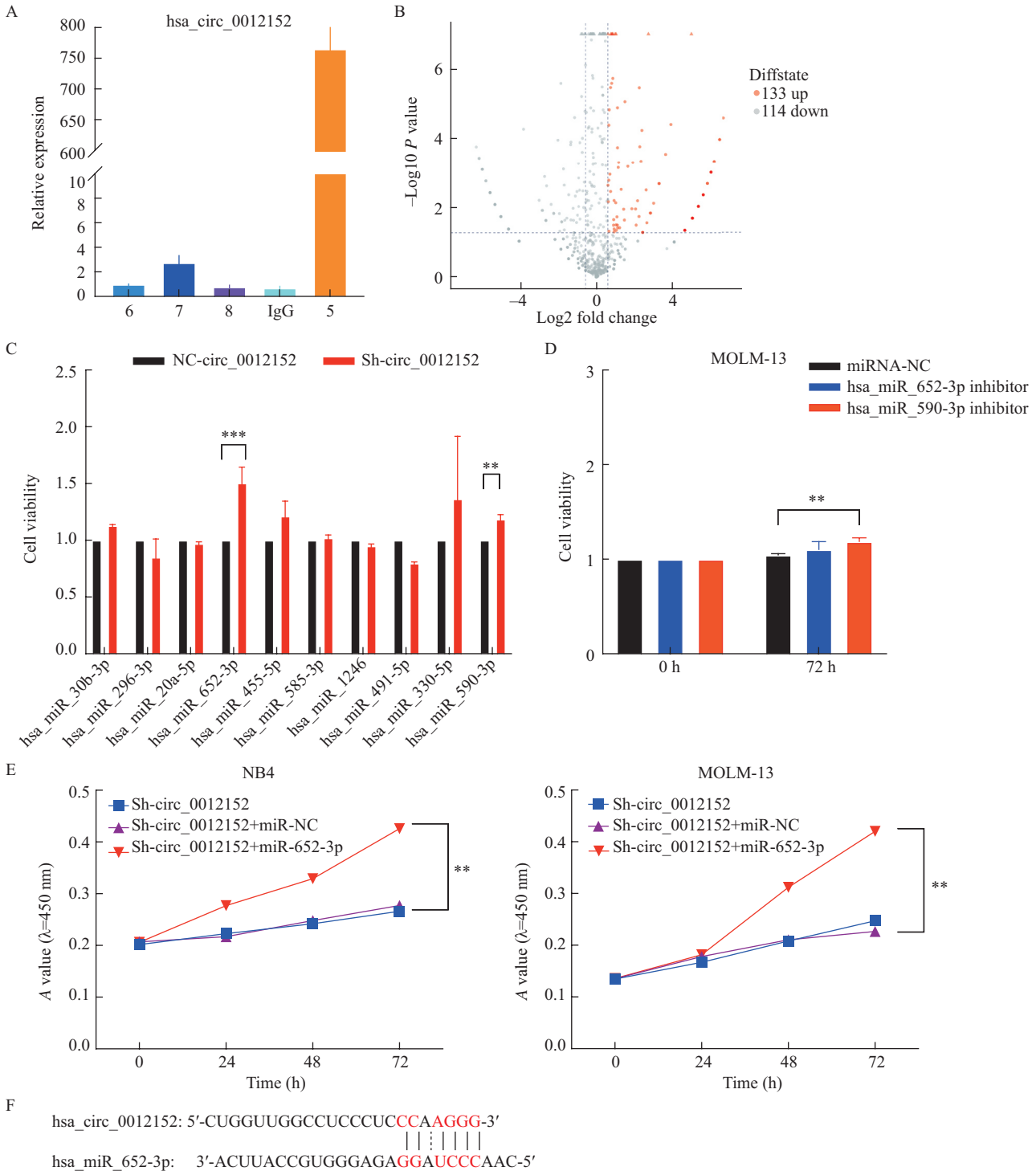


Fig. 3 Circ_0012152 acts as a sponge regulating miR-652-3p

A: The circ_0012152 expression level was detected after pull-down. 6: hsa_circ_0012152+MS2-CP; 7: hsa_circ_0012152-MS2+NC; 8: hsa_circ_0012152+NC; IgG: hsa_circ_0012152-MS2+MS2-CP; 5: hsa_circ_0012152-MS2+MS2-CP. B: Volcano plot of differentially expressed miRNAs in the negative control group *vs*. the circRNA enrichment group. C: comparison of growth rates between the NC group and the circ_0012152-knockdown group after transfection of MOLM-13 cells with miRNA inhibitors. D: comparison of growth rate between the NC-miR group and groups transfected with miR-652-3p and miR-590-3p inhibitors in the circ_0012152 group in MOLM-13 cells. E: The expression level of circ_0012152 in MOLM-13 and NB4 cells after transfection with sh-circ_0012152 and the miR-652-3p inhibitor was measured by qRT-PCR. F: The targeted sequence of circ_0012152 combined with miR-652-3p was simulated by bioinformatics. $^{**}P<0.01$, $^{***}P<0.001$

3 DISCUSSION

Although circ_0012152 expression in AML has been reported in previous studies, we performed a systematic analysis of circ_0012152 in a larger sample of 247 patients with AML. We found that circ_0012152 expression was increased in samples from patients with AML, and its high expression was negatively associated with the outcomes of

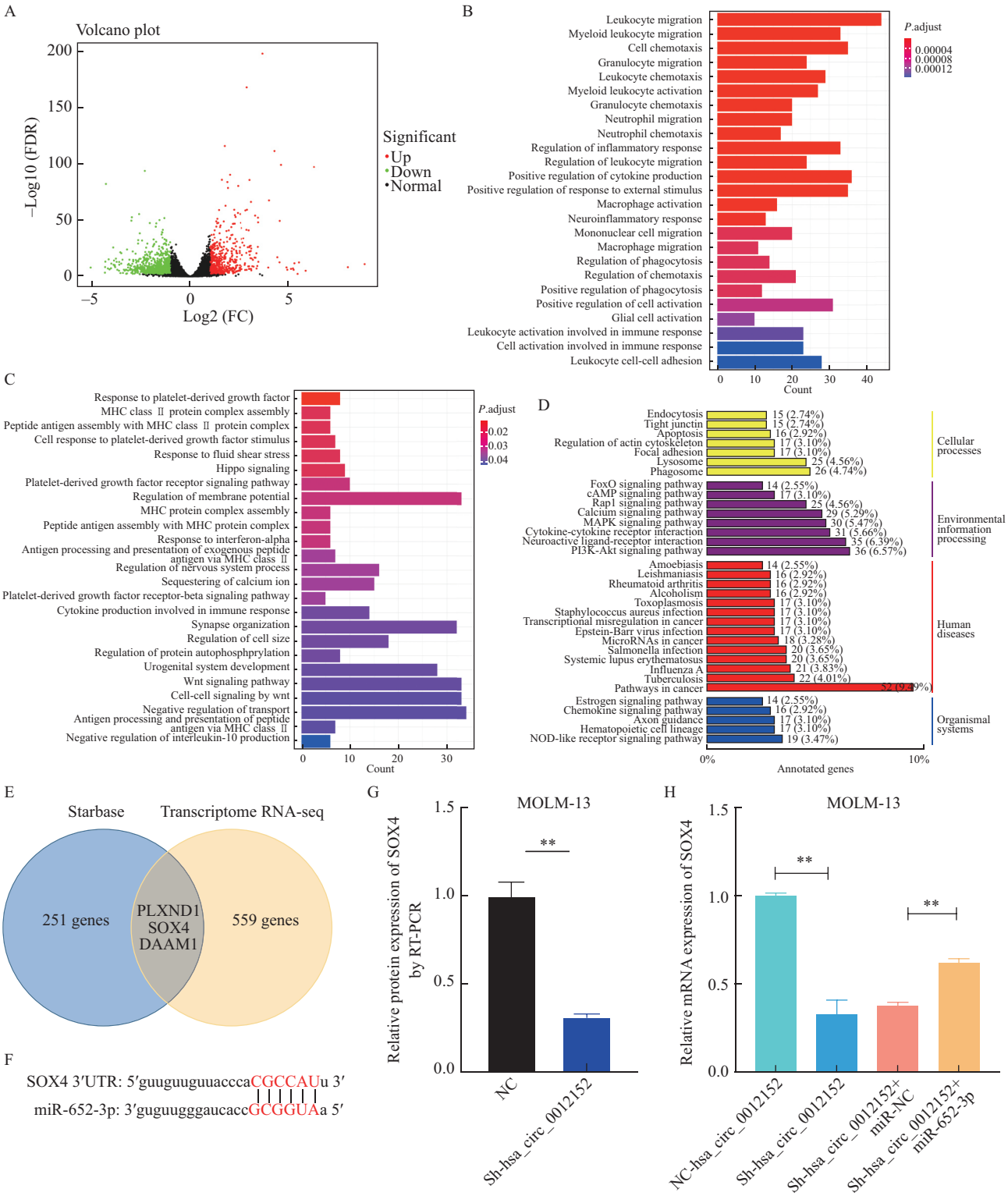


Fig. 4 MiR-652-3p regulated cell development by targeting SOX4 in AML cells

A: Volcano plot: differentially expressed mRNAs between the circ_0012152 knockdown group and NC group; B: GO functional annotation of upregulated differentially expressed genes; C: GO functional annotation of downregulated differentially expressed genes; D: KEGG functional annotation of target genes; E: intersection of starBase prediction and transcriptome RNA sequencing data for common target mRNAs; F: The binding sites between the SOX4 3'UTR and miR-652-3p were predicted by starBase; G: SOX4 expression level in circ_0012152-knockdown MOLM-13 cells; H: The expression level of SOX4 in MOLM-13 cells after transfection with sh-circ_0012152 and the miR-652-3p inhibitor was detected by RT-PCR. $^{**}P<0.01$

these patients, which is consistent with previous reports[20, 21]. We also observed that circ_0012152 expression was higher in patients with *FLT3-ITD* mutations than in those without *FLT3-ITD* mutations, and it is generally recognized that the *FLT3-ITD* mutation is an adverse predictor of AML[24, 25]. Moreover, circ_0012152 expression was higher in patients with non-APL AML than in those with APL, and its expression was positively correlated with the proportion

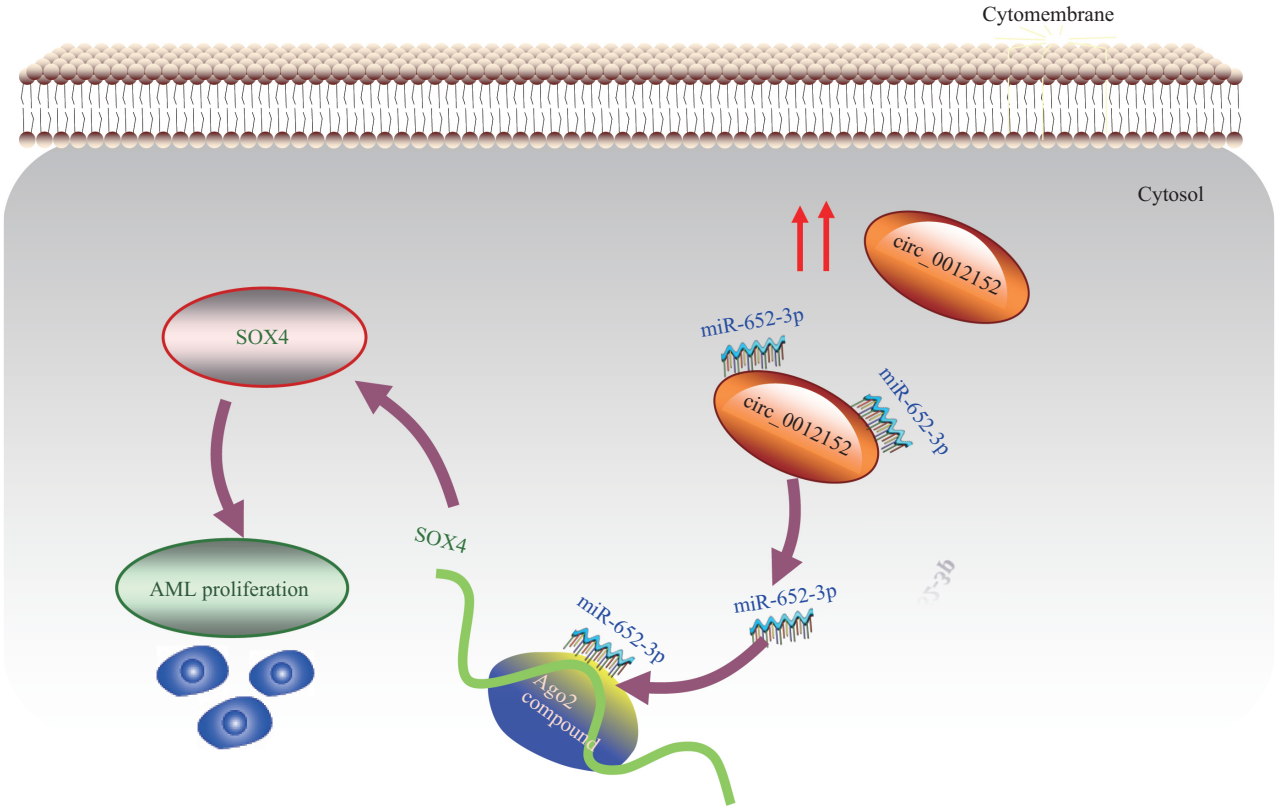


Fig. 5 The circ_0012152 upregulates SOX4 by "adsorbing" miR-652-3p in AML, serving as a new molecular mechanism involved in the occurrence and development of AML

of myeloblasts.

Interestingly, a previous study[26] indicated that circ_0012152 expression gradually decreased with the differentiation of hematopoietic stem cells. These findings suggest that circ_0012152 plays a role in the development of the myeloid lineage. In recent years, technologies, such as small interfering RNA (siRNA), antisense oligonucleotide (ASO), and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas13a, have been designed to target oncogenic circRNAs. Moreover, nanoparticles have numerous advantages, such as good biocompatibility, biodegradability, and high safety, which are all conducive to drug delivery[27]. Zhou *et al*[28] developed PLGA-based nanoparticles loaded with si-cSERPINE2, which effectively attenuated breast cancer progression *in vivo*. We believe that circ_0012152 may be a satisfactory therapeutic target in AML and that it could be applied as a clinical therapy through the abovementioned targeted drug delivery systems in the future.

To explore the molecular mechanism of circ_0012152, FISH was used to detect its subcellular localization. Circ_0012152 was primarily located in the cytoplasm, suggesting that it may act as a sponge to absorb miRNAs. According to the RNA pull-down results, the miR-652-3p inhibitor significantly relieved cell growth inhibition caused by circ_0012152 knockdown, which indicated that circ_0012152 might "absorb" miR-652-3p. The role of miR-652 in "tumor suppression" or as an "oncogene" varies among different tumors. In endometrial cancer, miR-652 is significantly upregulated, correlating with shorter OS and earlier recurrence, and overexpression of miR-652 promotes proliferation, migration, and invasion of endometrial cancer cells[29]. However, Chao *et al*[30] discovered that miR-652 inhibited the proliferation and metastasis of osteosarcoma cells by regulating *HOXA9*. Moreover, a previous study on pediatric ALL demonstrated that miR-652-3p was downregulated and that overexpression of miR-652-3p increased the sensitivity to vincristine and cytarabine and promoted apoptosis in Reh and RS4:11 cells[31]. Furthermore, Liu *et al*[21] reported that circ_0012152 might also act as an endogenous sponge of miR-30a, in turn inhibiting its activity, regulating the expression of MYSM1 and IER2, and promoting the proliferation of pediatric AML cells. Zhang *et al*[22] showed that circ_0012152 targeted miR-330-5p to increase the expression of *SOX4*, which promoted AML cell progression. Additionally, knockdown of circ_0012152 suppressed cell proliferation and promoted cell death by targeting *SOX12*, which was mediated by miR-625-5p in AML cells[20]. Therefore, it has been speculated that circ_0012152 binds to various miRNAs and plays biological roles through different pathways. However, in the present study, circ_0012152 was found to bind to miR-652-3p to regulate the transcription of target mRNAs, revealing a novel molecular mechanism of circ_0012152 that has not been reported in the literature.

Bioinformatics combined with transcriptome RNA sequencing revealed that *SOX4* was the most significantly downregulated gene in OCI-AML2 cells, which was also confirmed by qRT-PCR. Furthermore, the reduction in *SOX4* expression caused by circ_0012152 knockdown was attenuated by miR-652-3p inhibition. Thus, it was

hypothesized that circ_0012152 could exert its effects by "absorbing" miR-652-3p, in turn regulating *SOX4* expression. Several studies have reported that *SOX4* acts as an oncogene in various malignant tumors. For instance, *SOX4* not only plays a crucial role in the growth and metastasis of breast cancer but also binds to the CXCR7 promoter and upregulates the transcription of CXCR7[32]. In AML, patients with high *SOX4* expression exhibited lower remission rates and shorter OS. Overexpression of *SOX4* in zebrafish results in significant effacement and distortion of kidney structure due to increased myeloid progenitor cell infiltration[33]. Zhang *et al*[22] demonstrated that circ_0012152 affects *SOX4* expression. GO analysis of the downregulated DEGs indicated that these genes play a role in Wnt signaling. The Wnt signaling pathway has been highly conserved throughout evolution and is involved in important cell fate decisions during human development. Aberrant expression of Wnt pathway components, such as *WNT1*, *WNT2b* and *LEF-1*, provided evidence of the involvement of the Wnt/β-catenin pathway in the pathogenesis of AML[34]. Moreover, Tickenbrock *et al*[35] demonstrated abnormal activation of Wnt signaling in patients with AML with *FLT3-ITD*. Another study showed that SOX4 interacted directly with β-catenin and activated Wnt signaling[36]. Interestingly, our results showed that the expression of circ_0012152 increases in patients with *FLT3-ITD* mutations. Therefore, we speculate that Wnt signaling might be a downstream target of circ_0012152.

KEGG analysis of the transcriptome RNA sequencing data showed that the DEGs were most enriched in the PI3K/Akt signaling pathway in environmental information processing. The PI3K/Akt pathway regulates cell proliferation, apoptosis, and the cell cycle in hematological malignancies, including AML[37]. Mehta *et al*[38] identified *SOX4* as a mediator of PI3K/Akt signaling in breast cancer. Moreover, Ramezani-Rad *et al*[39] confirmed that SOX4 bound to and transcriptionally activated promoters of multiple components within the PI3K/Akt and MAPK signaling pathways, and they also demonstrated a role for *SOX4* in regulating PI3K activity in Ph+ ALL. Furthermore, we focused on exploring the downstream targets of circ_0012152/miR-652-3p/SOX4 and validating the association of circ_0012152 with the PI3K/Akt signaling pathway in AML.

In conclusion, circ_0012152 is overexpressed in patients with AML, and its high expression could be an independent prognostic factor. Circ_0012152 knockdown in the MOLM-13 and OCI-AML2 cell lines inhibited cell growth, increased apoptosis, reduced *CDK4*/*CDK6* expression, and induced cell cycle arrest at the G0/G1 phase. Circ_0012152 may act as a sponge for miR-652-3p, subsequently promoting *SOX4* expression.

Conflict of Interest Statement

The authors declare no competing interests.

REFERENCES

1 Papaemmanuil E, Gerstung M, Bullinger L, *et al*. Genomic Classification and Prognosis in Acute Myeloid Leukemia. N Engl J Med, 2016,374(23):2209-2221
2 Burnett A, Stone R. AML: New Drugs but New Challenges. Clin Lymphoma Myeloma Leuk, 2020,20(6):341-350
3 Thol F, Ganser A. Treatment of Relapsed Acute Myeloid Leukemia. Curr Treat Options Oncol, 2020,21(8):66
4 Cummins KD, Gill S. Will CAR T-cell therapy have a role in AML? Promises and pitfalls. Seminars in hematol, 2019,56(2):155-163
5 Fennell KA, Bell CC, Dawson MA. Epigenetic therapies in acute myeloid leukemia: where to from here? Blood, 2019,134(22):1891-1901
6 Kumar S, Gonzalez EA, Rameshwar P, *et al*. Non-Coding RNAs as Mediators of Epigenetic Changes in Malignancies. Cancers (Basel), 2020,12(12):3657
7 Jeck WR, Sorrentino JA, Wang K, *et al*. Circular RNAs are abundant, conserved, and associated with ALU repeats. RNA, 2013,19(2):141-157
8 Verduci L, Strano S, Yarden Y, *et al*. The circRNA-microRNA code: emerging implications for cancer diagnosis and treatment. Mol Oncol, 2019,13(4):669-680
9 Li A, Wang WC, McAlister V, *et al*. Circular RNA in colorectal cancer. J Cell Mol Med, 2021, 25(8):3667-3679
10 Lei B, Tian Z, Fan W, *et al*. Circular RNA: a novel biomarker and therapeutic target for human cancers. Int J Med Sci, 2019,16(2):292-301
11 Fu L, Tang D, Sun A, *et al*. Recent progress in study of circRNAs and its role in leukemia. J Leukoc Biol, 2021,109(4):731-739
12 Ding Y, Dong Y, Lu H, *et al*. Circular RNA profile of acute myeloid leukemia indicates circular RNA annexin A2 as a potential biomarker and therapeutic target for acute myeloid leukemia. Am J Transl Res, 2020,12(5):1683-1699
13 Hu Q, Gu Y, Chen S, *et al*. Hsa_circ_0079480 promotes tumor progression in acute myeloid leukemia via miR-654-3p/HDGF axis. Aging (Albany NY), 2020,13(1):1120-1131
14 Zhou X, Zhan L, Huang K, *et al*. The functions and clinical significance of circRNAs in hematological malignancies. J Hematol Oncol, 2020,13(1):138
15 Li W, Zhong C, Jiao J, *et al*. Characterization of hsa_circ_0004277 as a New Biomarker for Acute Myeloid Leukemia via Circular RNA Profile and Bioinformatics Analysis. Int J Mol Sci, 2017,18(3):597
16 Rybak-Wolf A, Stottmeister C, Glažar P, *et al*. Circular RNAs in the Mammalian Brain Are Highly Abundant, Conserved, and Dynamically Expressed. Mol Cell, 2015,58(5):870-885
17 Kristensen LS, Hansen TB, Venø MT, *et al*. Circular RNAs in cancer: opportunities and challenges in the field. Oncogene, 2018,37(5):555-565
18 Li Z, Huang C, Bao C, *et al*. Exon–intron circular RNAs regulate transcription in the nucleus. Nat Struct Mol Biol, 2015,22(3):256-264
19 Guo S, Li B, Chen Y, *et al*. Hsa_circ_0012152 and Hsa_circ_0001857 Accurately Discriminate Acute Lymphoblastic Leukemia From Acute Myeloid Leukemia. Front Oncol, 2020,10(1655):1-13
20 Shang Z, Ming X, Wu J, *et al*. Downregulation of circ_0012152 inhibits proliferation and induces apoptosis in acute myeloid leukemia cells through the miR-625-5p/SOX12 axis. Hematol Oncol, 2021,39(4):539-548
21 Liu X, Liu X, Cai M, *et al*. CircRNF220, not its linear cognate gene RNF220, regulates cell growth and is associated with relapse in pediatric acute myeloid leukemia. Mol Cancer, 2021,20(1):139
22 Zhang Z, Lin S, Yin J, *et al*. CircRNF220 plays a pathogenic role to facilitate cell progression of AML in vitro by sponging miR-330-5p to induce upregulation of SOX4. Histol Histopathol, 2022,37(10):1019-1030
23 Bataller A, Garrido A, Guijarro F, *et al*. European LeukemiaNet 2017 risk stratification for acute myeloid leukemia: validation in a risk-adapted protocol. Blood Adv, 2022,6(4):1193-1206

24 De Kouchkovsky I, Abdul-Hay M. Acute myeloid leukemia: a comprehensive review and 2016 update. Blood Cancer J, 2016,6(7):e441
25 Levis M. FLT3 mutations in acute myeloid leukemia: what is the best approach in 2013? Hematology Am Soc Hematol Educ Program, 2013,2013:220-226
26 Nicolet BP, Engels S, Aglialoro F, *et al*. Circular RNA expression in human hematopoietic cells is widespread and cell-type specific. Nucleic Acids Res, 2018,46(16):8168-8180
27 Zhang F, Jiang J, Qian H, *et al*. Exosomal circRNA: emerging insights into cancer progression and clinical application potential. J Hematol Oncol, 2023,16(1):67
28 Zhou B, Mo Z, Lai G, *et al*. Targeting tumor exosomal circular RNA cSERPINE2 suppresses breast cancer progression by modulating MALT1-NF-κB-IL-6 axis of tumor-associated macrophages. J Exp Clin Cancer Res, 2023,42(1):48
29 Sun X, Dongol S, Qiu C, *et al*. miR-652 Promotes Tumor Proliferation and Metastasis by Targeting RORA in Endometrial Cancer. Mol Cancer Res, 2018,16(12):1927-1939
30 Chao Y, Yang C, Xiong W, *et al*. miR-652 Inhibits the Proliferation, Migration, and Invasion of Osteosarcoma Cells by Targeting HOXA9 and Regulating the PI3K/Akt Signaling Pathway. J Oncol, 2022,2022:4809312
31 Jiang Q, Lu X, Huang P, *et al*. Expression of miR-652-3p and Effect on Apoptosis and Drug Sensitivity in Pediatric Acute Lymphoblastic Leukemia. Biomed Res Int, 2018,2018:5724686
32 Zhang J, Xiao C, Feng Z, *et al*. SOX4 promotes the growth and metastasis of breast cancer. Cancer Cell Int, 2020,20:468
33 Lu JW, Hsieh MS, Hou HA, *et al*. Overexpression of SOX4 correlates with poor prognosis of acute myeloid leukemia and is leukemogenic in zebrafish. Blood Cancer J, 2017,7(8):e593
34 Gruszka AM, Valli D, Alcalay M. Wnt Signaling in Acute Myeloid Leukemia. Cells, 2019, 8(11):1403
35 Tickenbrock L, Schwäble J, Wiedehage M, *et al*. Flt3 tandem duplication mutations cooperate with Wnt signaling in leukemic signal transduction. Blood, 2005,105(9):3699-3706
36 Moreno CS. SOX4: The unappreciated oncogene. Semin Cancer Biol 2020,67(Pt 1):57-64
37 Qu Y, Wang Y, Wang P, *et al*. Overexpression of long noncoding RNA HOXA-AS2 predicts an adverse prognosis and promotes tumorigenesis via SOX4/PI3K/AKT pathway in acute myeloid leukemia. Cell Biol Int, 2020,44(8):1745-1759
38 Mehta GA, Parker JS, Silva GO, *et al*. Amplification of SOX4 promotes PI3K/Akt signaling in human breast cancer. Breast Cancer Res Treat, 2017,162(3):439-450
39 Ramezani-Rad P, Geng H, Hurtz C, *et al*. SOX4 enables oncogenic survival signals in acute lymphoblastic leukemia. Blood, 2013,121(1):148-155

(Received Nov. 16, 2023; accepted Apr. 3, 2024)