Circ_0000005 Facilitates Proliferation, Apoptosis, Migration and Invasion of Acute Myeloid Leukemia Cells via Modulating miR-139-5p/Tspan3 Axis

Juan Tong
The First Affiliated Hospital of University of Science and Technology of China
Huilan Liu
The First Affiliated Hospital of University of Science and Technology of China
Changcheng Zheng
The First Affiliated Hospital of University of Science and Technology of China
Xiaoyu Zhu
The First Affiliated Hospital of University of Science and Technology of China
Xiang Wan
The First Affiliated Hospital of University of Science and Technology of China
Baolin Tang
The First Affiliated Hospital of University of Science and Technology of China
Wen Yao
The First Affiliated Hospital of University of Science and Technology of China
Lei Zhang
The First Affiliated Hospital of University of Science and Technology of China
Kaidi Song
The First Affiliated Hospital of University of Science and Technology of China
Guangyu Sun
The First Affiliated Hospital of University of Science and Technology of China
Zimin Sun (✉ hab518131@163.com)
The First Affiliated Hospital of University of Science and Technology of China https://orcid.org/0000-0001-9068-5071

Research

Keywords: Circ_0000005, AML, MiR-139-5p, Tspan3

Posted Date: August 25th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-57994/v1

Abstract

Background: Accumulating circular RNAs (circRNAs) are reported to be abnormally expressed in diverse cancers, hematologic malignancies included. This study aimed to investigate the biological function and underlying mechanisms of circ_0000005 in acute myeloid leukemia (AML).

Materials and methods: Bone marrow samples were enrolled from AML patients with normal samples as controls. Circ_0000005, miR-139-5p and tetraspanin 3 (Tspan3) were detected by qRT-PCR and Western blot, respectively. AML cell lines (KG1 and HL60) were used as cell models. CCK-8, Transwell and flow cytometry assays were adopted to study the biological functions of circ_0000005 on AML cells *in vitro*. The interrelation between circ_0000005 and miR-139-5p was detected by bioinformatics, qRT-PCR, luciferase reporter gene assay, RNA pull-down assay, and RNA-binding protein immunoprecipitation (RIP) assays. Ultimately, Western blot, qRT-PCR, luciferase reporter gene assay were adopted to corroborate the interrelation between miR-139-5p and its target Tspan3.

Results: Circ_0000005 was demonstrably elevated in both AML clinical samples and cell lines. Circ_0000005 overexpression promoted the viability, migration and invasion of AML cells, and repressed the apoptosis of AML cells, while silencing circ_0000005 showed opposite biological effects. Circ_0000005 interacted with miR-139-5p and repressed its expression, and Tspan3 was proved to be negatively regulated by miR-139-5p. Circ_0000005 could promote the expression of Tspan3 via repressing miR-139-5p, and the oncogenic functions of circ_0000005 were dependent on its regulatory function on miR-139-5p/Tspan3 axis.

Conclusion: Circ_0000005 facilitates the malignant phenotypes of AML cells via miR-139-5p/Tspan3 axis. Circ_0000005 may serve as a potential therapeutic target in AML.

1. Introduction

Acute myeloid leukemia (AML), characterized with high heterogeneity and rapid growth of abnormal primitive and infantile medullary cells in the bone marrow and peripheral blood, is a kind of hematological malignancy ensuing from clonal transformation of hematopoietic precursors (1, 2). Although great progresses have been made in the treatment of AML, primary or secondary chemoresistance is common in clinical practice, leading to unfavorable prognosis of the patients (2, 3). It is urgent to unmask the mechanism of AML progression for developing novel therapeutic targets.

Circular RNAs (circRNAs), are non-coding RNAs, and they possess covalently closed-loop structures derived from back splicing between the 3 'and 5' ends (4, 5). CircRNA is a vital participant in various physiological and pathological processes (6). The dysregulation of circRNAs are also implicated in diverse cancers (7). For example, circ_0000218 expression is dramatically up-regulated in colorectal cancer, which is in positive correlation with T stage and local lymph node metastasis (8). CircMAN2B2 promotes hepatocellular carcinoma progression via activating MAPK1 (9). CircPIP5K1A facilitates gastric cancer cell proliferation, invasion, migration and epithelial-mesenchymal transition (EMT) via activating

KRT80 and PI3K/AKT signaling (10). However, the biological functions of circRNA in AML are rarely reported.

MicroRNAs (miRNAs) block the translation of target genes via base-pairing with complementary sequences in the 3'-untranslated regions (3'-UTR) of target mRNAs (11–14). MiRNAs are crucial regulators in cancer biology. For example, miR-365a-3p modulates ADAM10-JAK-STAT signaling in colorectal cancer to block cancer progression (15). MiR-488 serves as a tumor suppressor in ovarian cancer via promoting tumor suppressor p53 expression (16). MiR-30a-5p contributes to the proliferation of cholangiocarcinoma cells through repressing SOCS3 (17). Notably, miR-139-5p impedes the malignant biological behaviors of several types of cancers, such as gastric cancer, cervical cancer, and AML (18–20).

The tetraspanin (tetraspan or TM4SF) family is a large group of integral membrane proteins with four transmembrane helices, during which 33 mammalian cell type-specific members were involved in the formation of plasma membrane signaling complexes (21, 22). Tspan3, belonging to TM4SF family, is verified to be associated with the malignancy of AML (23). Nevertheless, the upstream mechanism triggering the dysregulation of Tspan3 in AML awaits more clarification.

In the present work, we provided evidence that the expression of circ_0000005, a novel circRNA, was increased in AML samples and cell lines. Functionally and mechanistically, it was proved that circ_0000005 could sequester miR-139-5p to boost Tspan3 expression, and facilitate the proliferation, migratio and invasion of AML cells. Thus, it was concluded that circ_0000005 could serve as a promising therapeutic target for AML patients.

2. Materials And Methods

2.1 Tissue samples and ethics statement

Bone marrow samples were obtained from patients with newly diagnosed AML (n = 39) and healthy subjects (n = 39), from Department of Hematology, the First Affiliated Hospital of USTC. The monocytes were available from these samples through standard Ficoll-Hypaque density gradient centrifugation. This research was conducted with informed consent from all participators and endorsed by the Ethics Committee of the First Affiliated Hospital of USTC, University of Science and Technology of China.

2.2 Cell culture

The China Center for Type Culture Collection (CCTCC, Wuhan, China) was the provider of human AML cell lines (KG1, NB4, NOMO-1, and HL60) and bone marrow hematopoietic stem cell line (CD34). Aforementioned cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA), 100 U/ml penicillin, 100 μg/ml streptomycin (Hyclone, Logan, UT, USA) and L-glutamine (2 mM; Gibco, Carlsbad, CA, USA) in 5% CO_2 at 37 °C.

2.3 Treatment with RNase R and qRT-PCR

Firstly, total RNA extracted from tumor tissues and cells by Trizol reagent (Takara Biotechnology Co., Ltd., Dalian, China) was incubated with or without 3 U/mg RNAse R (Epicentre Technologies, Madison, WI, USA) for 20 min at 37 °C. Secondly, first-strand cDNA was generated adopting the Prime-Script RT reagent kit (TaKaRa, Dalian, China). Thirdly, qRT-PCR was operated on the StepOne Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc., Foster City, CA, USA) by the SYBR Green PCR kit (Takara Bio, Inc., Otsu, Japan). In this context, GAPDH or U6 was employed as endogenous controls. The primers' sequences were shown in Table 1.

Table 1
Sequences used for qRT-PCR.

circ_0000005	**F: 5'-AGGGGGAGCTGAGAGATCA-3'**
	R: 5'-TCTTTCCGAGACATTTGCTG-3'
miR-139-5p	F: 5'- GCCTCTACAGTGCACGTGTCTC-3'
	R: 5'- CGCTGTTCTCATCTGTCTCGC-3'
Tspan3	F: 5'-CATGTGATCTGGGCCG-3'
	R: 5'-CGCCAGTGATGAGGAG-3'
U6	F: 5'-CTCGCTTCGGCAGCACA-3'
	R: 5'-AACGCTTCACgAATTTGCGT-3'
GAPDH	F: 5'-ACACCCACTCCTCCACCTTT-3'
	R: 5'-TTACTCCTTGGAGGCCATGT-3'
Abbreviation: F stands for forward; R stands for reverse.	

2.4 Subcellular fractionation

Cells were lysed in the RIPA lysis (Solarbio, Beijing, China) and incubated for on ice 10 min. The cell lysate was separated by high-speed centrifugation (3 min, 12000 g). Cytoplasmic RNA was obtained in the supernatant and nuclear RNA in the nuclear pellet. Purified RNAs were estimated by qRT-PCR. Specifically, GAPDH and U6 worked as cytoplasmic and nuclear endogenous references, respectively.

2.5 Cell transfection

pcDNA3.1-circ_0000005, pcDNA3.1 empty vector, siRNA specific to circ_0000005, control siRNA (si-NC), miR-139-5p mimics and inhibitors (miR-139-5p and miR-139-5p in), mimics control and inhibitors control (miR-NC and NC inhibitors) were synthesized by Ribobio (Guangzhou, China). AML cells were transfected with the vectors or oligonucleotides mentioned above using Lipofectamine™ 3000 Transfection Reagent (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) in compliance with the manufacturer's instruction.

2.6 Cell counting kit-8 (CCK-8 assay)

After transfectoin, AML cells were inoculated onto 96 well plates (2000 cells in 100 μL serum medium / well). AT different time points (1, 2, 3 and 4 h after inoculation), 10 μL CCK-8 solution (Dojindo, Tokyo, Japan) was dripped into each well. After the cells were incubated for 1 h at room temperature, the absorbance of each well at 450 nm was assessed by a microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.7 Flow cytometry analysis

Cells rinsed with pre-cooled phosphate buffer saline (PBS) were resuspended in 500 μL binding buffer, and then stained with 5 μL fluorescein isothiocyanate (FITC)-Annexin-V (Sigma-Aldrich, Louis, MO, USA) staining solution and 10 μL propidium iodide (PI) staining solution (50 μg/mL, BD Biosciences, San Diego, CA, USA) for 15 min, away from light. Next, the cell apoptosis was estimated by the a flow cytometer (FACScan®; BD Biosciences, San Diego, CA, USA). The data were processed with FlowJo software (TreeStar, San Carlos, CA, USA).

2.8 Migration and invasion assays

Transwell chambers (pore size, 8 μm; Corning, NY, USA) coated with or without Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were used for invasion and migration assays, respectively. In brief, cell suspension in FBS-free RPMI-1640 medium (2×10^4 cells / well) and RPMI-1640 medium containing with 20% FBS (600 μL / well) were added into the upper and lower chambers, respectively. The cells were cultured for 48 h in 5% CO_2 at 37 ℃, and thereafter, migrated and invaded cells were recorded.

2.9 Western blot assay

RIPA lysis (Solarbio, Beijing, China) was adopted to isolate the total proteins from cells. The protein concentrations were quantified with BCA Protein Assay Kit (Beyotime, Haimen, China). The protein samples were then mixed with loading buffer, and heated in boiling water for 5 min for denaturation. After that 20 μg protein in each group was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After that, the membranes were blocked with 5% skim milk, and then incubated with diluted primary antibodies including anti-Tspan3 (ab151299, 1:1000, Abcam, China) and anti-GAPDH (ab8245, 1: 2000, Abcam, China) for 12 h at 4 ℃. After that, the membrane was incubated with HRP-conjugated secondary antibody (ab205718, 1:2000, Abcam) at room temperature at room temperature for 2 h. Finally, the protein bands were visualized by Enhanced Chemiluminescence Western Blotting Substrate (Dongguan Biotech, Shandong, China).

2.10 Luciferase reporter gene assay

Wild type (WT) and mutant (MUT) fragments of circ_0000005 sequences were inserted into pMIR-REPORT vectors (Promega, Madison, WI, USA). Similarly, WT and MUT fragments of Tspan3 3’-UTR with the binding sites for miR-139-5p were cloned into pMIR-REPORT vectors. After the reporter vectors were

constructed, the cells were co-transfected with these reporter vectors and miR-139-5p mimics or miR-NC. 48 h later, dual-luciferase reporter gene assay system (Promega, Madison, WI, USA) was employed to detect the luciferase activity in the cell lysate of each group.

2.11 RNA pull-down assay

Purified RNAs were labeled employing biotin with Pierce™ RNA 3'End Desthiobiotinylation Kit (Life Technologies, Baltimore, MD, USA). Cells transfected with biotin-labeled miR-139-5p (Bio-miR-139-5p) (GenePharma, Shanghai, China) or biotinylated RNAs (NC-Bio) (20 nM) by Lipofectamine™ 3000 (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) were harvested. The cells were added with lysis buffer, and then incubated on ice for 5 min. After that, the lysis was centrifuged and the cell debris was discarded. After that, magnetic beads were was added and incubated at 4 °C for 2 h and then RNA was purified by RNeasy Mini Kit (QIAGEN, Duesseldorf, Germany). Ultimately, qRT-PCR was performed to detect the expression of circ_0000005.

2.12 RNA immunoprecipitation (RIP) assay

The Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) was applied to RIP assay. In short, RIPA lysis buffer (Beyotime, Shanghai, China) was employed to lyse T-ALL cells, and 200 μL cell lysates were incubated with 5 μg antibody against anti-Argonaute 2 (Ago 2) (Abcam, Cambridge, UK) or anti-immunoglobulin G (IgG) (Abcam, Cambridge, UK) and rotated at 4 °C overnight. After being treated with proteinase K (Beyotime, Shanghai, China), immunoprecipitated RNA was extracted by the RNeasy MinElute Cleanup Kit (Qiagen, Shanghai, China) and reversely transcribed adopting Prime-Script RT Master Mix (TaKaRa, Dalian, China). Eventually, qRT-PCR was utilized to detect the abundance of circ_000005 and miR-139-5p.

2.13 Statistical analysis

Data were analyzed on the basis of IBM SPSS 17.0 software (IBM, Armonk, NY, USA) and GraphPad Prism 7.0 (GraphPad Software, Inc. La Jolla, CA, USA), and showed in the form of mean ± SD. The difference between groups was observed by Student's *t*-test, $\chi 2$ test or Wilcoxon test. The correlations among circ_0000005, miR-139-5p and Tspan3 expressions in AML samples was evaluated through Pearson's correlation analysis.

3. Results

3.1 Circ_0000005 expression was markedly up-regulated in AML

Above all, we detected the expression of circ_0000005 in bone marrow samples from 39 AML patients and 39 healthy volunteers. The data of qRT-PCR unveiled that circ_0000005 expression was dramatically enhanced in AML tissues in comparison to in control tissues (Fig. 1A). Besides, we measured circ_0000005 expression levels in human AML cell lines and bone marrow hematopoietic stem cell line by qRT-PCR, and found that circ_0000005 expression was greatly higher in AML cells (Fig. 1B). Next, RNA was isolated from the RNase R-treated KG1 and HL60 cells, and qRT-PCR proved that GAPDH levels decreased notably by the RNase R treatment, but no significant change was observed on circ_0000005 (Fig. 2A). This implied that circular structure of circ_0000005. Additionally, subcellular fractionation assay indicated that most of circ_0000005 were located in the cytoplasm of AML cells (Fig. 2B). These data unveiled that circ_0000005 might participate in gene expression regulation at post-transcriptional level.

3.2 Circ_0000005 facilitated proliferation, migration and invasion, and repressed apoptosis of AML cells

To expound the biological function of circ_0000005 in the development of AML, we adopted pcDNA-circ_0000005 to up-regulate circ_0000005 expression level in KG1 cells and employed circ_0000005 siRNAs to decrease circ_0000005 expression level in HL60 cells (Fig. 3A). CCK-8 and Transwell assays highlighted that the circ_0000005 promoted the proliferation, migration and invasion of KG1 cells (Fig. 3B-D). Subsequently, we assessed the role of circ_0000005 on apoptosis of AML cells and observed that the rate of apoptosis of KG1 cells was markedly lower in circ_0000005 overexpression group (Fig. 3E-F). Conversely, silencing circ_0000005 repressed the proliferation, migration, invasion, promoted the apoptosis of HL-60 cells (Fig. 3B-F). In brief, the aforementioned results demonstrated that circ_0000005 contributed to promoting the malignant phenotypes of AML cells.

3.3 Circ_0000005 sponged miR-139-5p in AML cells

The target miRNAs of circ_0000005 were predicted through CircInteractome database, and the bioinformatics analysis implied a miRNA response element between circ_0000005 and miR-139-5p (Fig. 4A). Next, we constructed luciferase reporter vectors containing wild type (WT) or mutant type (MUT) circ_0000005 sequence, and miR-139-5p was co-transfected with the luciferase reporters into KG1 and HL60 cells. Dual-luciferase reporter gene assay uncovered that miR-139-5p markedly reduced relative luciferase activity of WT reporter, but didn’t repress the relative luciferase activity of MUT reporter (Fig. 4B). Furthermore, RIP assay also authenticated that miR-139-5p could interact with circ_0000005 directly (Fig. 4C). Additionally, RNA pull-down assay confirmed that circ_0000005 was enriched in the complex containing biotin-miR-139-5p (Fig. 4D-E). Furthermore, qRT-PCR indicated that circ_0000005 negatively modulated miR-139-5p expression in AML cells (Fig. 4F). Moreover, miR-139-5p expression was declined in AML tissues in comparison with in normal bone marrows; it was also repressed in AML cell lines as against in bone marrow hematopoietic stem cell line (Fig. 4G, H). Collectively, these results suggested that miR-139-5p was a downstream target of circ_0000005, and could be negatively regulated by it in AML cells.

3.4 MiR-139-5p inhibited the malignancy of AML cells

To delve into the biological function of miR-139-5p in AML, miR-139-5p mimics and inhibitors were transferred into AML cells (Fig. 5A). *In vitro* experiments showed that HL60 cell proliferation, migration and invasion were restrained by miR-139-5p overexpression, while cell apoptosis was promoted (Fig. 5B-F). Conversely, the opposite effect was observed after KG1 cells were transfected with miR-139-5p inhibitors (Fig. 5B-F). These findings depicted that miR-139-5p, as a tumor suppressor, could probably impede the progression of AML cells. Next, we transfected miR-139-5p mimics into KG1 cells with circ_0000005 overexpression (Fig. 6A), and found that transfection with miR-139-5p mimics rescued the promoting effect of circ_0000005 overexpressing on cell proliferation, migration and invasion, as well as the inhibitory effect on cell apoptosis (Fig. 6B-F). These findings suggested that the circ_0000005/ miR-139-5p axis figured prominently in regulating AML progression.

3.5 Tspan3 was directly targeted by miR-139-5p in AML cells

To explore the molecular mechanism through which miR-139-5p worked on AML cells, bioinformatics analysis was adopted to identify the potential target of miR-139-5p, and thereafter, it was noticed that Tspan3 3'-UTR contained a binding sequence for miR-139-5p (Fig. 7A). Then dual-luciferase reporter gene assay was performed, the results of which showed that miR-139-5p overexpression markedly reduced luciferase activity of WT Tspan3 3'-UTR, while miR-139-5p mimics didn't change the luciferase activity on MUT Tspan3 3'UTR reporter (Fig. 7B). Moreover, qRT-PCR and Western blot assays also underscored that miR-139-5p mimics markedly restrained Tspan3 mRNA and protein expression levels in AML cells, whereas inhibition of miR-139-5p expression boosted Tspan3 expression (Fig. 7C-D). Additionally, miR-139-5p mimics counterfeited the promoting effect on Tspan3 expression induced by circ_0000005 overexpression (Fig. 7E). After that, we measured circ_0000005, miR-139-5p and Tspan3 mRNA expressions in AML samples and proved that circ_0000005 was negatively correlated to miR-139-5p (Fig. 8A). The expression of miR-139-5p was also verified to be negatively correlated with Tspan3 expression (Fig. 8B), while circ_0000005 was in positive association with Tspan3 expression (Fig. 8C). These findings verified that Tspan3 was a downstream of miR-139-5p in AML, and it could be positively regulated by circ_0000005.

4. Discussion

Accumulating evidence reveals that circRNAs are characterized by highly conserved sequence and high degree of stability (24). CircRNAs originate from abnormal splicing or non-linear reverse splicing (6, 25). CircRNAs are cell- and tissue-specific, as well as well-known as promising biomarkers for the diagnosis of cancers (26). Accumulating studies authenticate that circRNAs are crucial in the progression of malignancies via modulating gene expressions, including AML (27). For example, circ_0000370 expression is upregulated in FLT3-ITD + AML, which promotes cell proliferation and inhibits cell apoptosis (28). CircRNA-DLEU2 serves as an oncogenic circRNA in the AML progression via promoting PRKACB

expression (29). Circ-Foxo3 is in positive association with the Foxo3 gene expression and its high expression contributes to a better prognosis of AML patients (30). In this study, we first proved that circ_0000005 expression was greatly elevated in AML. Moreover, circ_0000005 overexpression markedly facilitated the malignant biological behaviors of AML cells. These demonstrations implied that circ_0000005 worked as a novel oncogenic circRNA in AML, which could probably be a potential therapy target. Nonetheless, it still remains to be explored whether it can be used as a biomarker to evaluate/predict disease progression and the prognosis of the AML patients in the following work.

MiR-139-5p is reported to be tumor suppressor in multiple types of cancers. In gastric cancer, it negatively regulates SLC39A7 and modulates Akt/mTOR signaling signaling to repress cancer cell proliferation and migration (31). In cervical cancer, miR-139-5p represses TCF4 and inhibits Wnt/β-catenin signaling to block cancer progression (19). MiR-139-5p refrains the viability of normal CD34(+)-hematopoietic stem and progenitor cells, and interferes with the *in vitro* differentiation of myeloid cells (32). Additionally, miR-139-5p constrains cancer cell proliferation, invasion and migration, and induces cell cycle arrest at the S phase in AML cells (20). In this study, it was demonstrated that miR-139-5p expression was declined in AML samples, and we verified that miR-139-5p overexpression in AML cells evidently constrained cell proliferation, migration, and invasion, and enhanced apoptosis, whereas transfection with miR-139-5p inhibitors worked oppositely. Our demonstrations are consistent with the previous.

It is worth noting that circRNA, abounding in miRNA binding sites, can serve as miRNA sponges, thereby regulating target gene expression (33). For example, circRGNEF promotes bladder cancer progression via sponging miR-548 to enhance KIF2C expression (34). Circ_0072387 sponges miR-503-5p to suppress proliferation, metastasis, and glycolysis of oral squamous cell carcinoma cells (35). In the present work, though online analysis database, we found a binding site between circ_0000005 and miR-139-5p. Luciferase reporter gene assay, RNA pull-down assay and RIP assay authenticated that circ_0000005 could adsorbed miR-139-5p, and the transfection with miR-139-5p mimics counterfeited the promotion of circ_0000005 overexpression on cell proliferation, migration and invasion, as well as the inhibitory effect on cell apoptosis. In brief, circ_0000005 took part in regulating the progression of AML cells via negatively modulating miR-139-5p expressions. This interaction also partly explained the mechanism of miR-139-5p dysregulation in AML.

Tetraspanins, a class of transmembrane proteins, are vital participants in the construction of the plasma membrane (36). Mounting reports demonstrate that multiple TM4SF family members facilitate tumor cell proliferation and metastasis (36, 37). For example, tetraspanin 1 facilitates epithelial-to-mesenchymal transition and metastasis of cholangiocarcinoma through binding to integrin α6β1 to activate the PI3K/AKT/GSK-3β/Snail/PTEN feedback loop (38). In lung cancer, Tspan8 overexpression promotes the proliferation of cancer cells; besides, silencing Tspan8 leads to G1 phase arrest and accelerates apoptosis by negatively regulating CDK2, CDK4, and Cyclin D1, and positively modulating Bax and PARP (39). Moreover, Tspan3 is verified to be a vital factor in multiple tumors. For example, Tspan3 functions as a proliferation-related factor, whose depletion represses colonic cancer cell proliferation (40). Furthermore, it is also proved that Tspan3 is a target of Musashi 2, a key coordinator of leukemia

progression, and the decreased Tspan3 prominently impairs the AML development (20). It's worth noting that, multiple TM4SF family members are negatively regulated by miRNA. For example, miR-378a-3p suppresses the progression of glioblastoma multiforme by reducing TSPAN17 expression (41). MiR-369-3p suppresses TSPAN13, thereby impeding the proliferation of thyroid papillary carcinoma cells (42). MiR-139-5p constrains cell proliferation, invasion and migration capabilities, and induces cell cycle arrest at the S phase in AML cells via negatively regulating Tspan3 (20). In the present work, we validated that miR-139-5p could negatively regulate Tspan3 expression; furthermore, we observed that circ_0000005 could also positively modulate Tspan3 expression in AML. Our data suggested that the competitive endogenous RNA (ceRNA) network formed by circ_0000005, miR-139-5p and Tspan3, was contributive to AML progression (Fig. 9).

5. Conclusion

In summary, our findings underline that circ_0000005 expression is up-regulated in AML and the promotive effects of circ_0000005 on AML development are closely associated with miR-139-5p and Tspan3. These findings may provide useful clues for the diagnosis and treatment of AML.

Declarations

Ethics approval and consent to participate

This research was conducted with informed consent from all participators and endorsed by the Ethics Committee of the First Affiliated Hospital of USTC, University of Science and Technology of China. All participants signed informed consent

Consent for publication

The study was undertaken with the patient's consent.

Availability of data and material

The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests

All authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contributions

Conceived and designed the experiments: JT, HLL, CCZ, XW, BLT, WY, KDS, ZMS;

Performed the experiments: JT, HLL, XYZ, XW, BLT, LZ, GYS, ZMS;

Statistical analysis: JT, GYS, ZMS;

Wrote the paper: JT, XW, BLT, GYS, ZMS.

All authors read and agreed to this final manuscript.

Acknowledgements

Not applicable.

References

1. Ping L, Jian-Jun C, Chu-Shu L, Guang-Hua L, Ming Z. Silencing of circ_0009910 inhibits acute myeloid leukemia cell growth through increasing miR-20a-5p. Blood cells, molecules & diseases. 2019;75:41-7.
2. Shang J, Chen WM, Wang ZH, Wei TN, Chen ZZ, Wu WB. CircPAN3 mediates drug resistance in acute myeloid leukemia through the miR-153-5p/miR-183-5p-XIAP axis. Experimental hematology. 2019;70:42-54.
3. Roma A, Spagnuolo PA. Estrogen Receptors Alpha and Beta in Acute Myeloid Leukemia. Cancers. 2020;12(4).
4. Li W, Yang FQ, Sun CM, Huang JH, Zhang HM, Li X, et al. circPRRC2A promotes angiogenesis and metastasis through epithelial-mesenchymal transition and upregulates TRPM3 in renal cell carcinoma. Theranostics. 2020;10(10):4395-409.
5. Guo SS, Li BX, Zou DB, Yang SJ, Sheng LX, Ouyang GF, et al. Tip of the iceberg: roles of circRNAs in hematological malignancies. American journal of cancer research. 2020;10(2):367-82.
6. Zhang HD, Jiang LH, Sun DW, Hou JC, Ji ZL. CircRNA: a novel type of biomarker for cancer. Breast cancer (Tokyo, Japan). 2018;25(1):1-7.
7. Bach DH, Lee SK, Sood AK. Circular RNAs in Cancer. Molecular therapy Nucleic acids. 2019;16:118-29.
8. Pei FL, Cao MZ, Li YF. Circ_0000218 plays a carcinogenic role in colorectal cancer progression by regulating miR-139-3p/RAB1A axis. Journal of biochemistry. 2020;167(1):55-65.
9. Fu X, Zhang J, He X, Yan X, Wei J, Huang M, et al. Circular RNA MAN2B2 promotes cell proliferation of hepatocellular carcinoma cells via the miRNA-217/MAPK1 axis. Journal of Cancer. 2020;11(11):3318-26.
10. Song H, Xu Y, Xu T, Fan R, Jiang T, Cao M, et al. CircPIP5K1A activates KRT80 and PI3K/AKT pathway to promote gastric cancer development through sponging miR-671-5p. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie. 2020;126:109941.

11. Emamgolizadeh Gurt Tapeh B, Mosayyebi B, Samei M, Beyrampour Basmenj H, Mohammadi A, Alivand MR, et al. microRNAs involved in T-cell development, selection, activation, and hemostasis. Journal of cellular physiology. 2020. doi: 10.1002/jcp.29689.
12. Ma X, Jin L, Lei X, Tong J, Wang R. MicroRNA3633p inhibits cell proliferation and induces apoptosis in retinoblastoma cells via the Akt/mTOR signaling pathway by targeting PIK3CA. Oncology reports. 2020;43(5):1365-1374.
13. Paiva RM, Zauli DAG, Neto BS, Brum IS. Urinary microRNAs expression in prostate cancer diagnosis: a systematic review. Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico. 2020. doi: 10.1007/s12094-020-02349-z.
14. Pottoo FH, Javed N, Rahman J, Abu-Izneid T, Khan FA. Targeted delivery of miRNA based therapeuticals in the clinical management of Glioblastoma Multiforme. Seminars in cancer biology. 2020;S1044-579X(20)30084-5. doi: 10.1016/j.semcancer.2020.04.001.
15. Hong YG, Xin C, Zheng H, Huang ZP, Yang Y, Zhou JD, et al. miR-365a-3p regulates ADAM10-JAK-STAT signaling to suppress the growth and metastasis of colorectal cancer cells. Journal of Cancer. 2020;11(12):3634-44.
16. Guo JY, Wang XQ, Sun LF. MicroRNA-488 inhibits ovarian cancer cell metastasis through regulating CCNG1 and p53 expression. European review for medical and pharmacological sciences. 2020;24(6):2902-10.
17. Zhang JW, Wang X, Li GC, Wang D, Han S, Zhang YD, et al. MiR-30a-5p promotes cholangiocarcinoma cell proliferation through targeting SOCS3. Journal of Cancer. 2020;11(12):3604-14.
18. Hou J, Zhuo H, Chen X, Cheng J, Zheng W, Zhong M, et al. MiR-139-5p negatively regulates PMP22 to repress cell proliferation by targeting the NF-kappaB signaling pathway in gastric cancer. International journal of biological sciences. 2020;16(7):1218-29.
19. Ji X, Guo H, Yin S, Du H. miR-139-5p functions as a tumor suppressor in cervical cancer by targeting TCF4 and inhibiting Wnt/beta-catenin signaling. OncoTargets and therapy. 2019;12:7739-48.
20. Zhang R, Tang P, Wang F, Xing Y, Jiang Z, Chen S, et al. Tumor suppressor miR-139-5p targets Tspan3 and regulates the progression of acute myeloid leukemia through the PI3K/Akt pathway. Journal of cellular biochemistry. 2019;120(3):4423-32.
21. Hemler ME. Tetraspanin functions and associated microdomains. Nature reviews Molecular cell biology. 2005;6(10):801-11.
22. Qi W, Sun L, Liu N, Zhao S, Lv J, Qiu W. Tetraspanin family identified as the central genes detected in gastric cancer using bioinformatics analysis. Molecular medicine reports. 2018;18(4):3599-610.
23. Kwon HY, Bajaj J, Ito T, Blevins A, Konuma T, Weeks J, et al. Tetraspanin 3 Is Required for the Development and Propagation of Acute Myelogenous Leukemia. Cell stem cell. 2015;17(2):152-64.
24. Hsu MT, Coca-Prados M. Electron microscopic evidence for the circular form of RNA in the cytoplasm of eukaryotic cells. Nature. 1979;280(5720):339-40.

25. Verduci L, Strano S, Yarden Y, Blandino G. The circRNA-microRNA code: emerging implications for cancer diagnosis and treatment. Molecular oncology. 2019;13(4):669-80.
26. Li Y, Zhao J, Yu S, Wang Z, He X, Su Y, et al. Extracellular Vesicles Long RNA Sequencing Reveals Abundant mRNA, circRNA, and lncRNA in Human Blood as Potential Biomarkers for Cancer Diagnosis. Clinical chemistry. 2019;65(6):798-808.
27. Yao Z, Luo J, Hu K, Lin J, Huang H, Wang Q, et al. ZKSCAN1 gene and its related circular RNA (circZKSCAN1) both inhibit hepatocellular carcinoma cell growth, migration, and invasion but through different signaling pathways. Molecular oncology. 2017;11(4):422-37.
28. Zhang L, Bu Z, Shen J, Shang L, Chen Y, Wang Y. A novel circular RNA (hsa_circ_0000370) increases cell viability and inhibits apoptosis of FLT3-ITD-positive acute myeloid leukemia cells by regulating miR-1299 and S100A7A. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie. 2020;122:109619.
29. Wu DM, Wen X, Han XR, Wang S, Wang YJ, Shen M, et al. Role of Circular RNA DLEU2 in Human Acute Myeloid Leukemia. Molecular and cellular biology. 2018;38(20).
30. Zhou J, Zhou LY, Tang X, Zhang J, Zhai LL, Yi YY, et al. Circ-Foxo3 is positively associated with the Foxo3 gene and leads to better prognosis of acute myeloid leukemia patients. BMC cancer. 2019;19(1):930.
31. Zhang Y, Bai J, Si W, Yuan S, Li Y, Chen X. SLC39A7, regulated by miR-139-5p, induces cell proliferation, migration and inhibits apoptosis in gastric cancer via Akt/mTOR signaling pathway. Bioscience reports. 2020;40(2).
32. Emmrich S, Engeland F, El-Khatib M, Henke K, Obulkasim A, Schoning J, et al. miR-139-5p controls translation in myeloid leukemia through EIF4G2. Oncogene. 2016;35(14):1822-31.
33. Li Y, Ge YZ, Xu L, Jia R. Circular RNA ITCH: A novel tumor suppressor in multiple cancers. Life sciences. 2019:117176.
34. Yang C, Li Q, Chen X, Zhang Z, Mou Z, Ye F, et al. Circular RNA circRGNEF promotes bladder cancer progression via miR-548/KIF2C axis regulation. Aging. 2020;12.
35. Han L, Cheng J, Li A. hsa_circ_0072387 Suppresses Proliferation, Metastasis, and Glycolysis of Oral Squamous Cell Carcinoma Cells by Downregulating miR-503-5p. Cancer biotherapy & radiopharmaceuticals. 2020. doi: 10.1089/cbr.2019.3371.
36. Beckwith KA, Byrd JC, Muthusamy N. Tetraspanins as therapeutic targets in hematological malignancy: a concise review. Frontiers in physiology. 2015;6:91.
37. Yang YG, Sari IN, Zia MF, Lee SR, Song SJ, Kwon HY. Tetraspanins: Spanning from solid tumors to hematologic malignancies. Experimental hematology. 2016;44(5):322-8.
38. Wang Y, Liang Y, Yang G, Lan Y, Han J, Wang J, et al. Tetraspanin 1 promotes epithelial-to-mesenchymal transition and metastasis of cholangiocarcinoma via PI3K/AKT signaling. Journal of experimental & clinical cancer research : CR. 2018;37(1):300.
39. Dong Z, Zhao L, Lu S, Xiong J, Geng Z. Overexpression of TSPAN8 Promotes Tumor Cell Viability and Proliferation in Nonsmall Cell Lung Cancer. Cancer biotherapy & radiopharmaceuticals.

2016;31(10):353-9.

40. Moss AC, Doran PP, Macmathuna P. In Silico Promoter Analysis can Predict Genes of Functional Relevance in Cell Proliferation: Validation in a Colon Cancer Model. Translational oncogenomics. 2007;2:1-16.
41. Guo XB, Zhang XC, Chen P, Ma LM, Shen ZQ. miR-378a-3p inhibits cellular proliferation and migration in glioblastoma multiforme by targeting tetraspanin 17. Oncology reports. 2019;42(5):1957-71.
42. Li P, Dong M, Wang Z. Downregulation of TSPAN13 by miR-369-3p inhibits cell proliferation in papillary thyroid cancer (PTC). Bosnian journal of basic medical sciences. 2019;19(2):146-54.

Figures

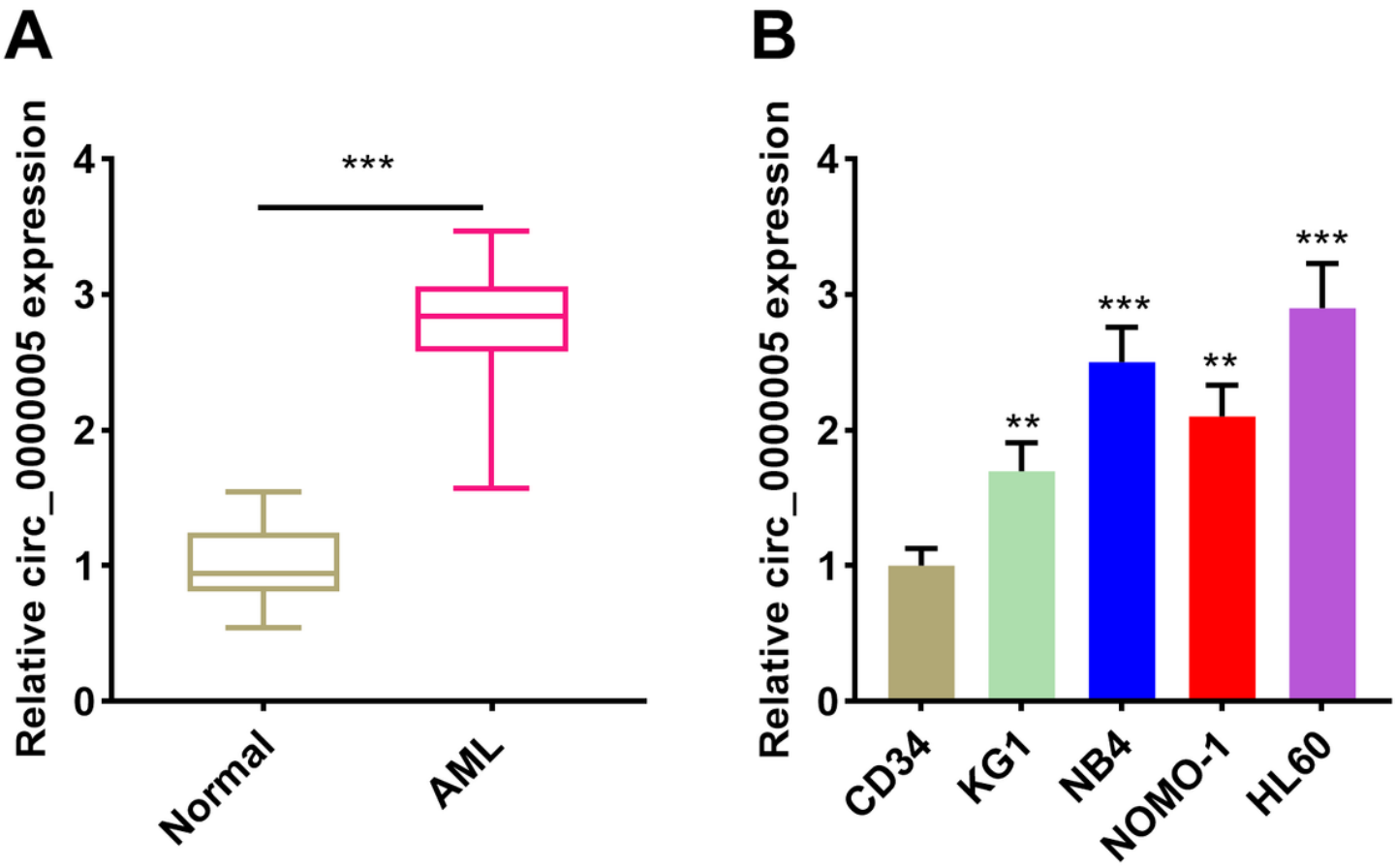

Figure 1

Circ_0000005 expression was up-regulated in AML. A. qRT-PCR was performed to measure the circ_0000005 expression levels in bone marrow samples from AML patients (n = 39) and healthy controls (n = 39). B. qRT-PCR was used to examine the expression of circ_0000005 in four human AML cell lines (KG1, NB4, NOMO-1, and HL60) and bone marrow hematopoietic stem cell line (CD34). * denotes P < 0.05, ** denotes P < 0.01 and *** denotes P < 0.001.

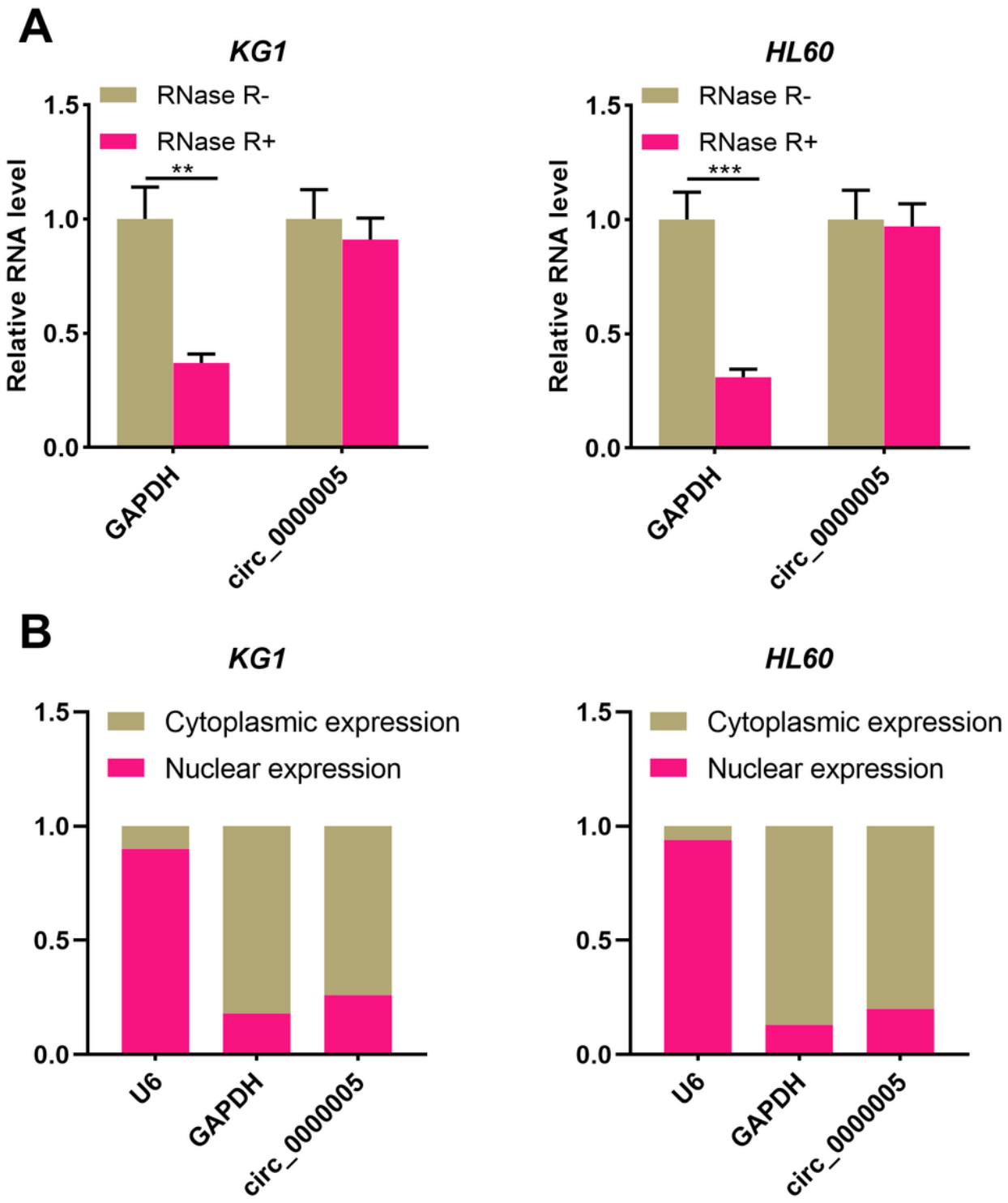

Figure 2

The biological characteristics of circ_0000005 in AML cell lines. A. The expressions of circ_0000005 and GAPDH in AML cells treated with or without RNase R were detected by qRT-PCR. B. Circ_0000005 expression levels in nucleus and cytoplasm were evaluated by subcellular fractionation assay. ** denotes $P < 0.01$ and *** denotes $P < 0.001$.

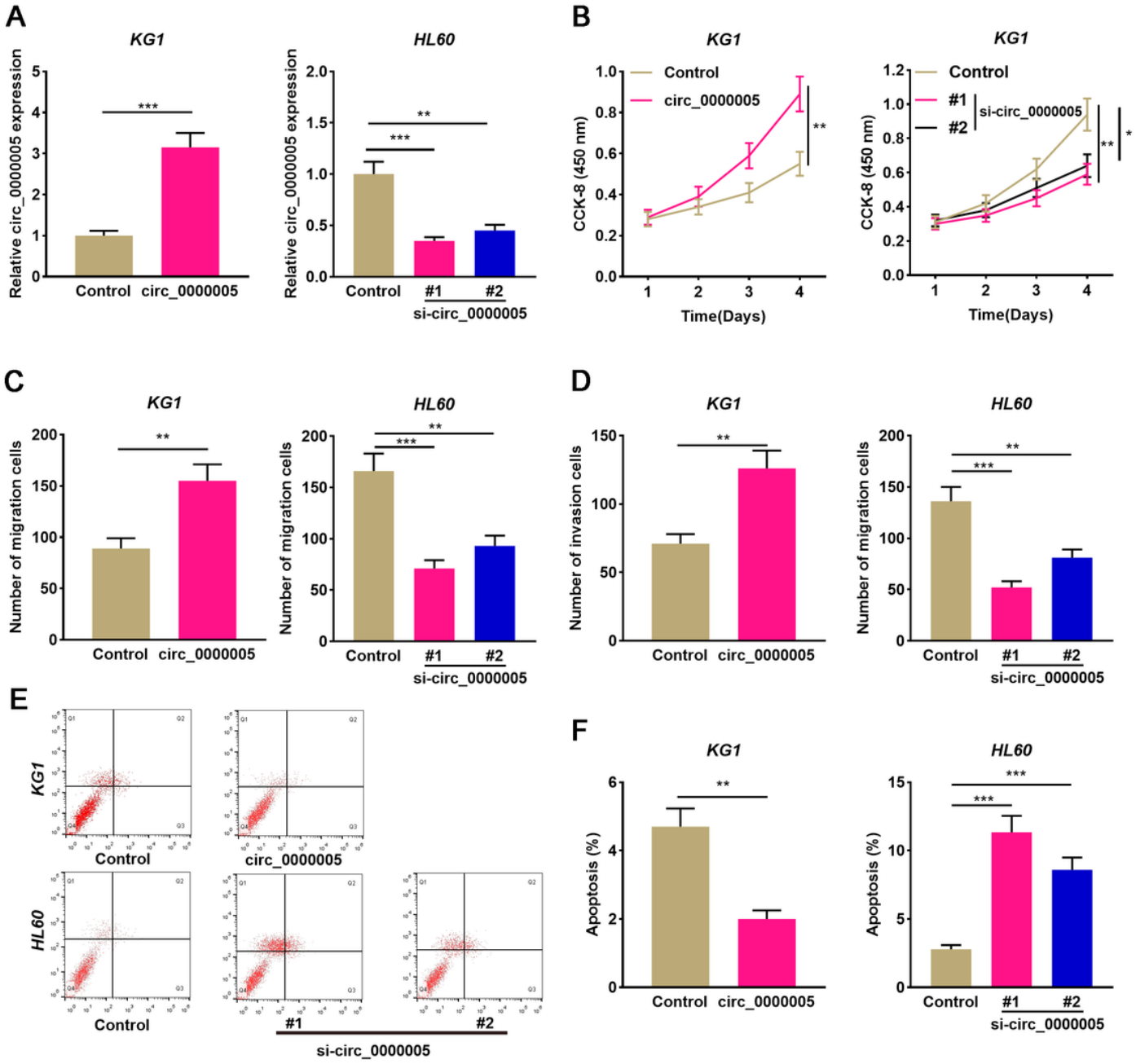

Figure 3

Effect of circ_0000005 on cell proliferation, migration, invasion and apoptosis of AML. A. KG1 and HL60 cells were transfected with circ_0000005 overexpressing vector (pcDNA-circ_0000005) or small interfering RNA (siRNA) against circ_0000005 (si-circ_0000005#1, circ_0000005#2), and then the expression of circ_0000005 was measured by qRT-PCR. B. Cell proliferation was assessed by CCK-8 assay. C-D. The migratory and invasive capability was assessed by Transwell assay. E-F. Cell apoptosis was detected by Annexin V-FITC/PI double staining method. ** denotes P < 0.01 and *** denotes P < 0.001.

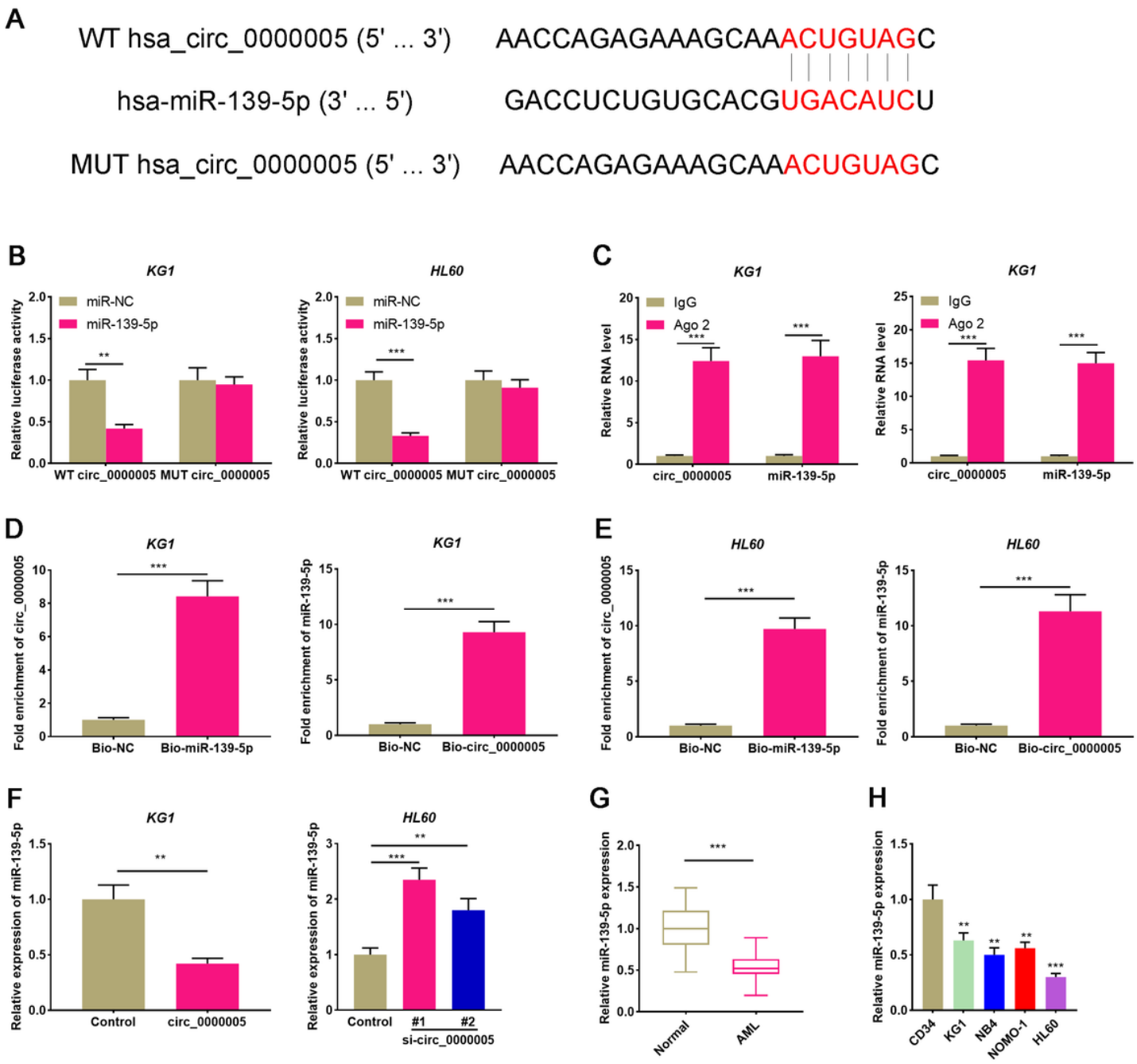

Figure 4

Circ_0000005 interacted with miR-139-5p. A. The binding sites between wide type (WT) circ_0000005 and miR-139-5p was predicted, and the mutant type (MUT) circ_0000005 sequence was designed. B. Dual-luciferase reporter assays showed that miR-139-5p mimics could repress the luciferase activity of circ_0000005-WT, rather than circ_0000005-MUT. C. RIP assay was performed to validate the direct interaction between circ_0000005 and miR-139-5p in AML cells. D-E. RNA Pull-down assay confirmed that circ_0000005 could directly bind to miR-139-5p. F. qRT-PCR showed that circ_0000005 could negatively regulate miR-139-5p expression in AML cells. G. MiR-139-5p expression level in bone marrows from AML

patients (n = 39) and healthy volunteers (n = 39) was determined by qRT-PCR. H. qRT-PCR was used to analyze the expression of miR-139-5p in four human AML cell lines (KG1, NB4, NOMO-1, and HL60) and bone marrow hematopoietic stem cell line (CD34). ** denotes P < 0.01 and *** denotes P < 0.001.

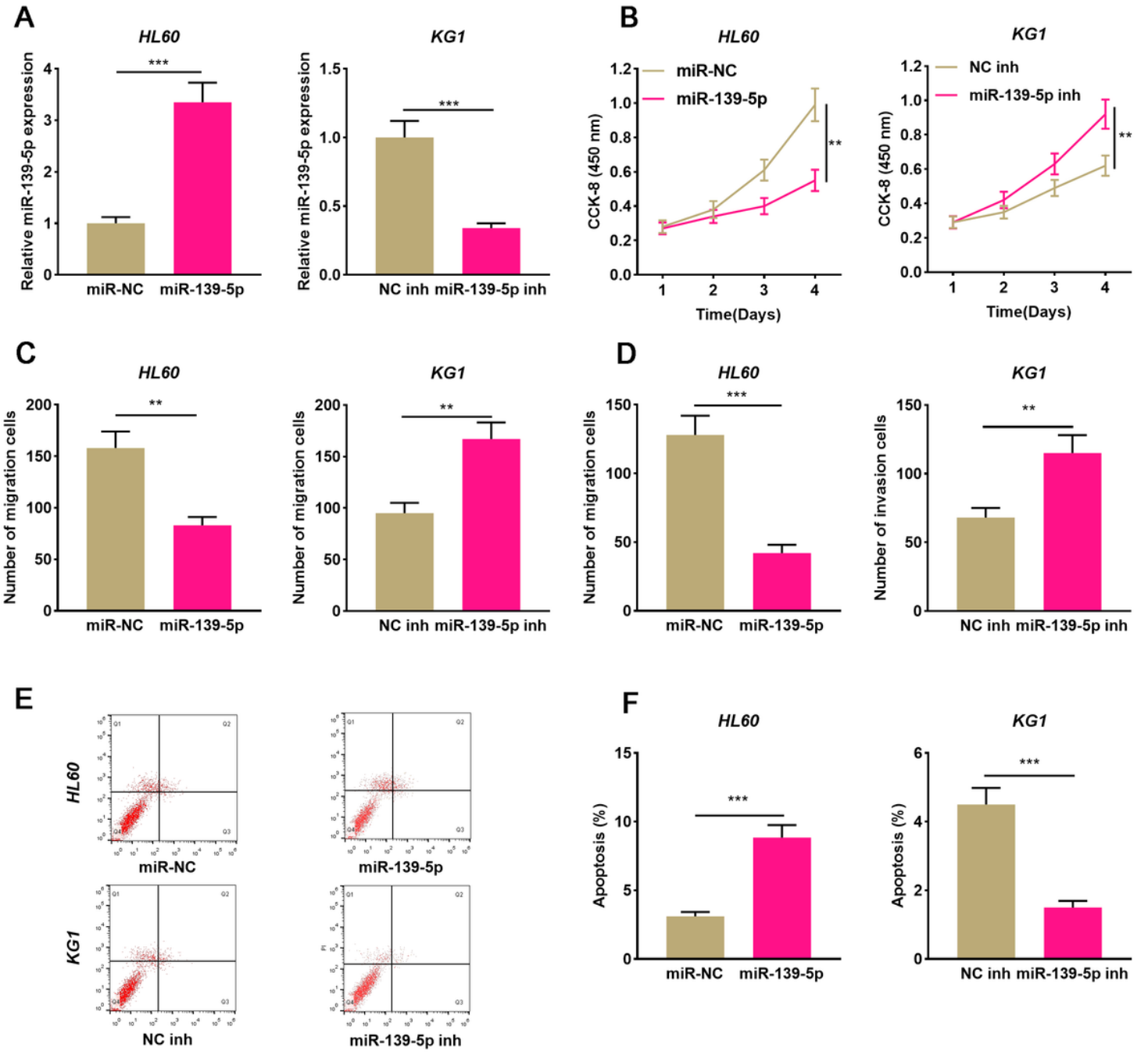

Figure 5

MiR-139-5p exerted tumor-suppressive roles in AML. A. HL60 and KG1 cells were transfected with miR-139-5p mimics and miR-139-5p inhibitors, and then the expression of miR-139-5p were detected by qRT-PCR. B. Cell proliferation was detected by CCK-8 assay. C-D. Cell migration and invasion were detected by Transwell assay. E-F. Cell apoptosis was detected by Annexin V-FITC/PI double staining method. ** denotes P < 0.01 and *** denotes P < 0.001.

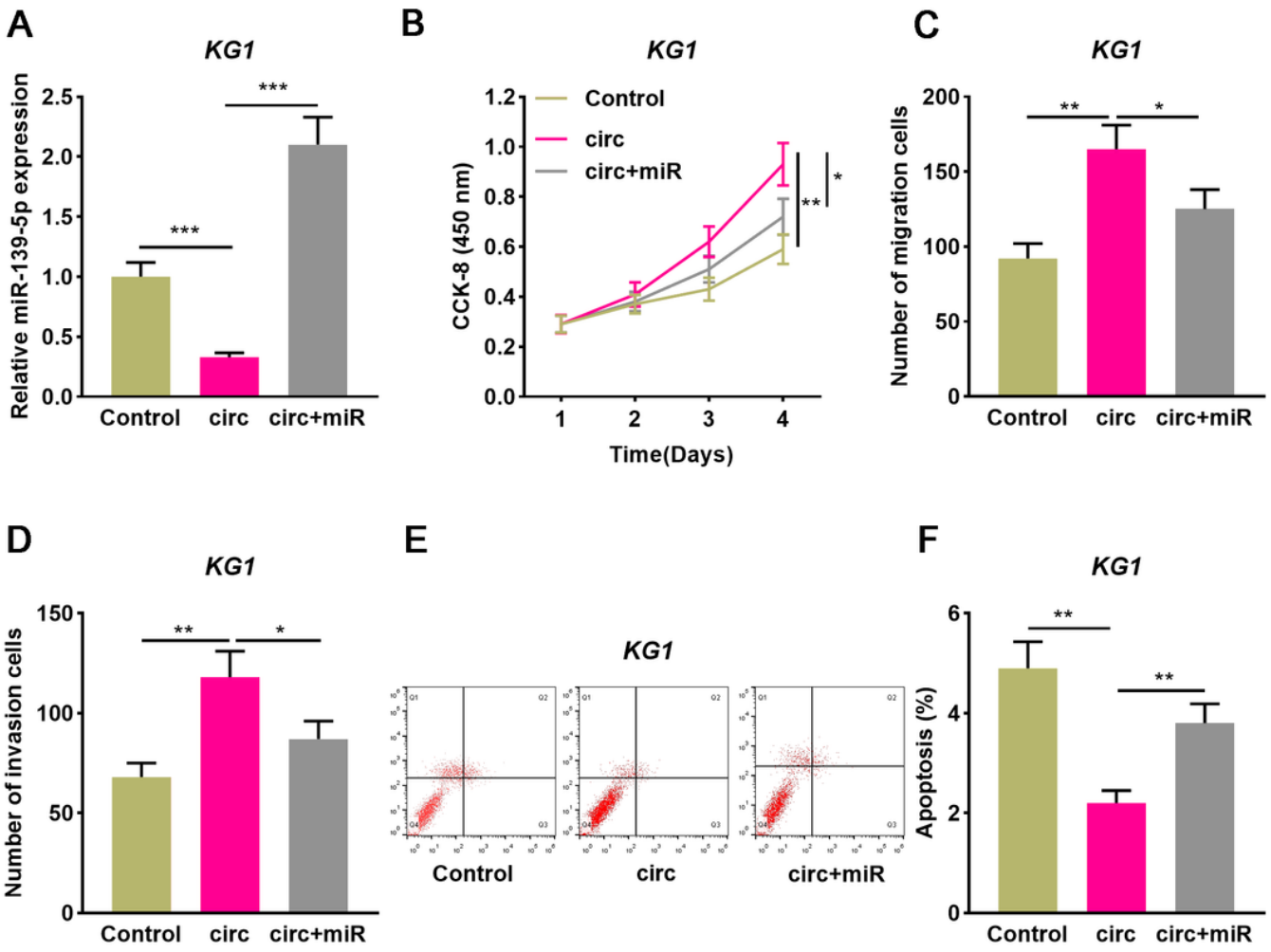

Figure 6

Restoration of miR-139-5p expression reversed the effects of circ_0000005 on AML cells. A. Circ_0000005- overexpressing HL60 cells were transfected with miR-139-5p mimics, and then the expression of miR-139-5p were detected by qRT-PCR. B. Cell proliferation was detected by CCK-8 assay. C-D. Cell migration and invasion were detected by Transwell assay. E-F. Cell apoptosis was detected by Annexin V-FITC/PI double staining method. * denotes $P < 0.05$, ** denotes $P < 0.01$ and *** denotes $P < 0.001$.

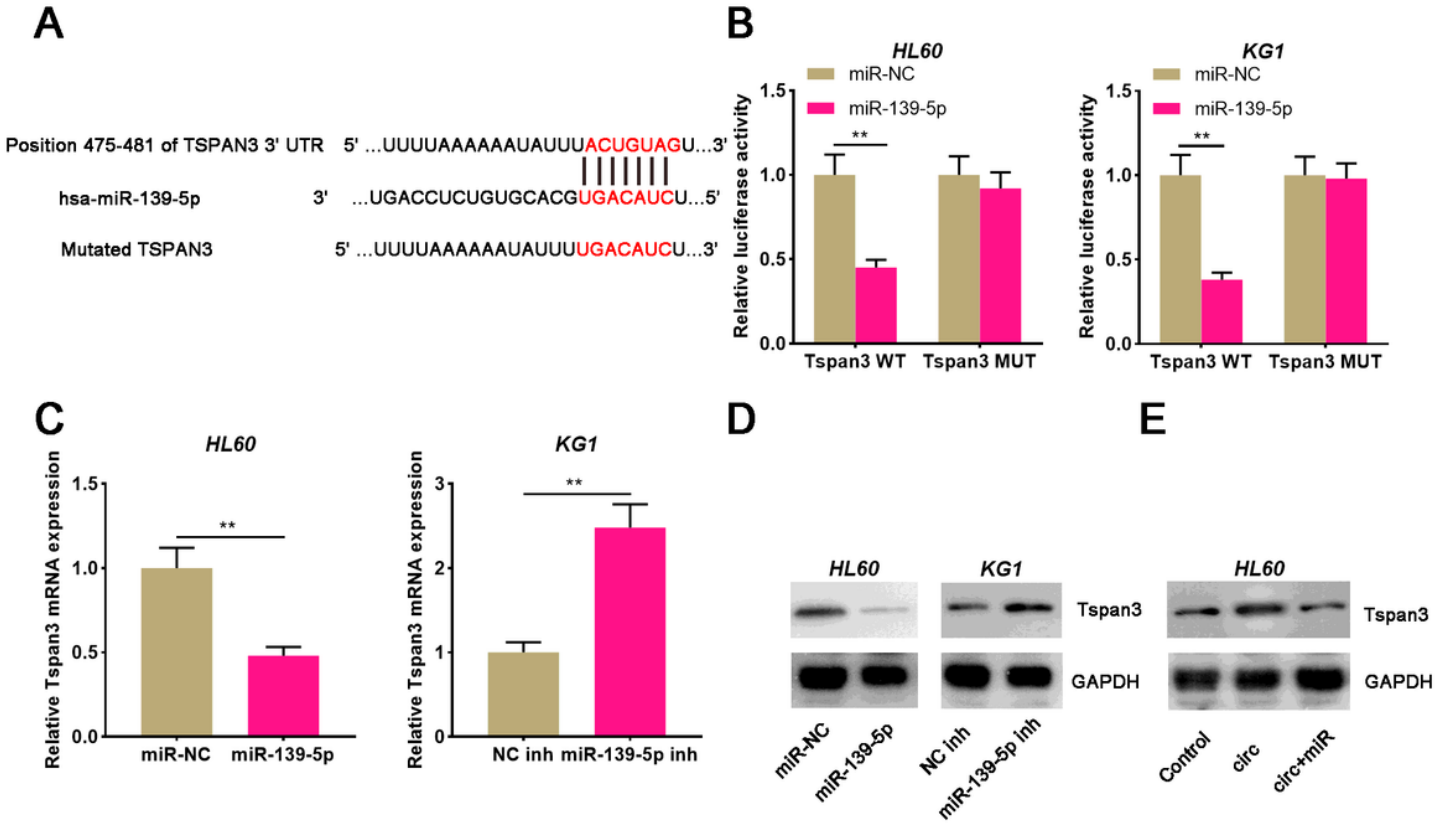

Figure 7

Tspan3 was a direct target of miR-139-5p in AML cells. A. Bioinformatic analysis through Targetscan showed that the 3'UTR of Tspan3 possessed a sequence that was complementary with miR-139-5p. B. Dual luciferase reporter assay showed that miR-139-5p overexpression inhibited the activity of luciferase carrying wild-type (WT) 3'-UTR of Tspan3, but had no effect on mutant (MUT) 3'-UTR of Tspan3. C-D. qRT-PCR and Western blot indicated that miR-139-5p overexpression reduced the expressions of Tspan3 mRNA and protein in HL60 cells and miR-139-5p inhibition increased the expression level of Tspan3 mRNA and protein in KG1 cells. E Western blot assay showed miR-139-5p mimics reversed the promotive effect on Tspan3 expression induced by circ_0000005 overexpression. ** denotes $P < 0.01$ and *** denotes $P < 0.001$.

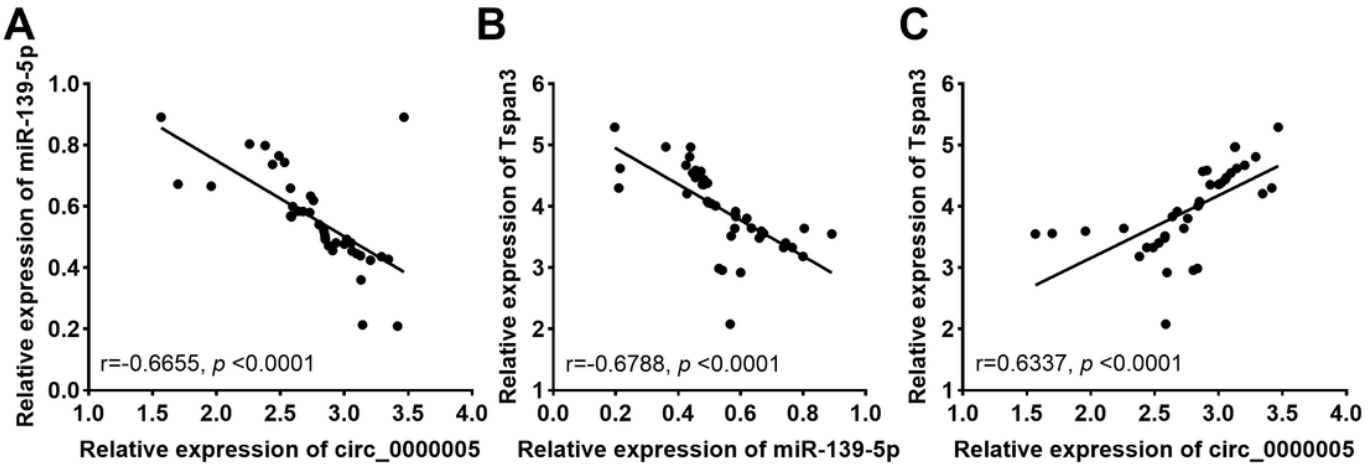

Figure 8

The correlations among the expression levels of circ_0000005, miR-139-5p and Tspan3. A. The expression level of circ_0000005 was negatively correlated with miR-139-5p expression in AML tissues. B. The expression level of miR-139-5p was negatively correlated with Tspan3 expression in AML tissues. C. The expression level of Tspan3 was positively correlated with circ_0000005 expression in AML tissues.

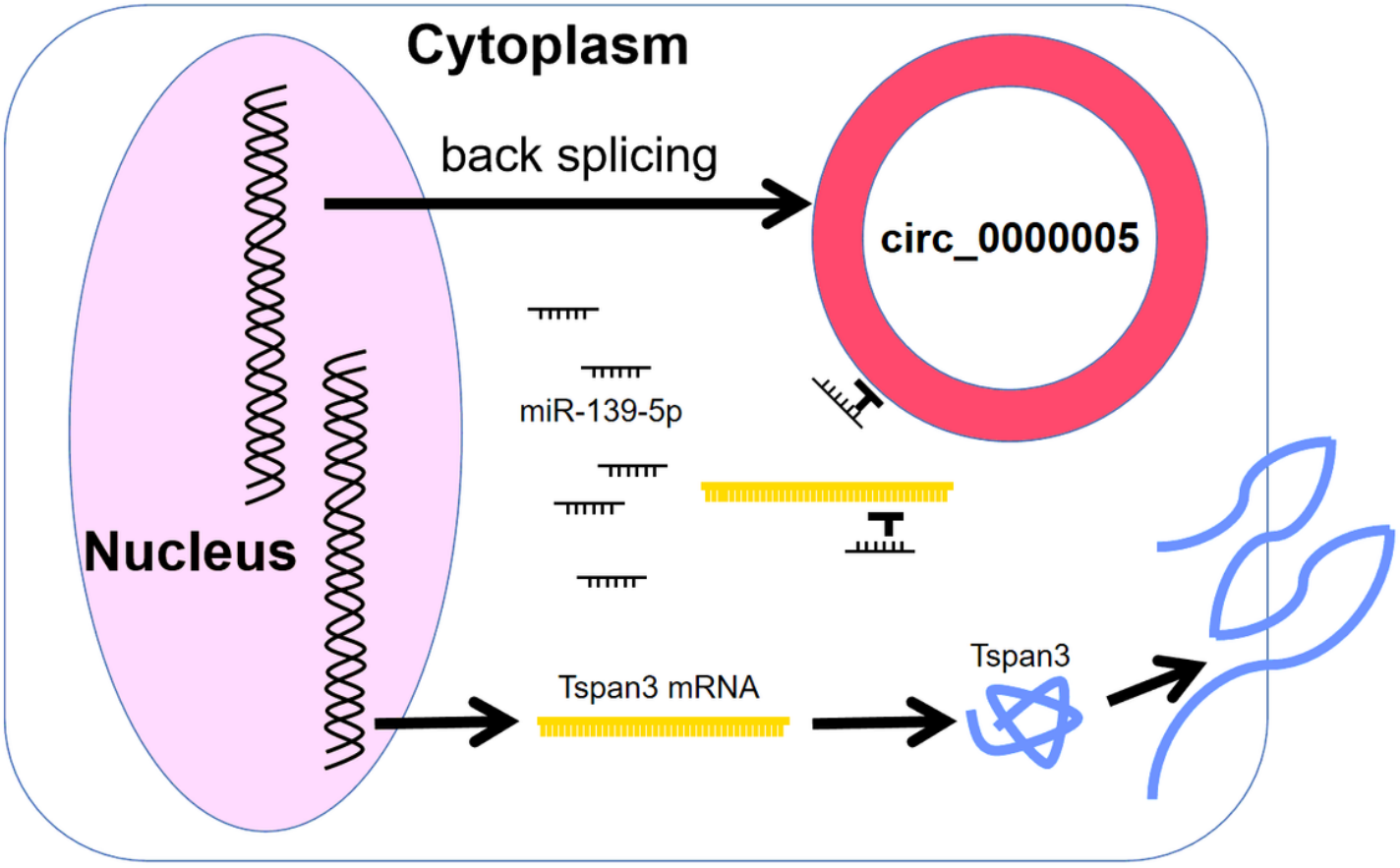

Figure 9

Graphic Abstract: circ_0000005 serves as a molecular sponge to repress miR-139-5p and up-regulate Tspan3, to promote the progression of AML