Circular RNA_0001073 (circ_0001073) Suppresses The Progression of Non-Small Cell Lung Cancer via miR-582-3p/RGMB Axis

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Abstract

Objective: Reportedly, circular RNAs (circRNAs) exert a crucial regulatory role in cancer. Circ_0001073 is derived from exons 3-5 of ACVR2A gene, which inhibits cancer progression. However, the role and mechanism of circ_0001073 in non-small cell lung cancer (NSCLC) are unclear. This study aimed to explore the role and mechanism of circ_0001073 in the development of NSCLC.

Materials and Methods: In this experimental study, microarray analysis was employed to filter differential expressed circRNAs in NSCLC tissues. Also, circ_0001073, microRNA-582-3p (miR-582-3p), and repulsive guidance molecule B (RGMB) mRNA expressions were examined by quantitative real-time polymerase chain reaction (qRT-PCR). NSCLC cell multiplication was measured by the cell counting kit-8 (CCK-8) assay. Scratch healing experiment and Transwell experiment were performed to assess cell migration and invasion, respectively. Flow cytometry was applied to analyze the apoptosis of NSCLC cells. Western blot was employed to assess RGMB protein expression. Additionally, dual-luciferase reporter gene experiment and RNA immunoprecipitation (RIP) experiment were applied to probe the binding sites between miR-582-3p and circ_0001073 or RGMB.

Results: Circ_0001073 was remarkably under-expressed in NSCLC tissues and cells. Circ_0001073 overexpression impeded the multiplication, migration, and invasion and enhanced the apoptosis of NSCLC cells in vitro. Circ_0001073 directly bound to miR-582-3p and acted as a miRNA sponge to regulate RGMB expression. Besides, miR-582-3p overexpression or knockdown of RGMB remarkably reversed the malignant phenotypes of NSCLC cells induced by the up-regulation of circ_0001073 expression.

Conclusion: Circ_0001073 up-regulates RGMB expression through adsorbing miR-582-3p to inhibit NSCLC progression, suggesting its potential as a novel therapeutic target in NSCLC.

Keywords: Circular RNA, MicroRNA, Non-Small Cell Lung Cancer, Repulsive Guidance Molecule B

Introduction

Lung cancer (LC) is a common and high-mortality disease and becomes the major cause of cancer-related deaths worldwide (1). Non-small cell lung cancer (NSCLC) is the main pathological type of LC, taking up 80-85% of all LC cases. Most patients with NSCLC are diagnosed at an advanced stage and suffer from an adverse prognosis, with a 5-year survival rate of less than 20% (2-4). It is imperative to discover novel and effective therapeutics for NSCLC.

Circular RNA (circRNA), an endogenous non-coding RNA molecule, is widely found in the eukaryotes. It has closed-loop structure and is more stable than linear RNA (5). CircRNAs are vital regulators in the development of diverse diseases, including cancers (6-11). For instance, circ_0006332 expression is up-modulated in bladder cancer tissues, and it regulates MYBL2 expression by working as a sponge for miR-143, thereby promoting bladder cancer progression (9). Reportedly, circ_0001073 expression is down-modulated in the lung adenocarcinoma (LUAD) tissues compared with normal lung tissues (10). Nevertheless, whether circ_0001073 regulates NSCLC progression remains largely unknown. Previous studies reported that miR-582-3p is abnormally expressed in different tumors, such as prostate cancer, acute myeloid leukemia, and NSCLC (12-14). A recent study reported that miR-582-3p enhances the cancer stem cell properties of NSCLC cells (14). However, the potential mechanism by which miR-582-3p regulates NSCLC has not been fully clarified.

Repulsive guidance molecule B (RGMB), also known as "Dragon", is the first discovered member of the RGM family (15). It is unveiled that RGMB expression is down-regulated in NSCLC, and knockdown of RGMB enhances the adhesion, migration, and invasion of NSCLC cells (16). This suggests that RGMB participates in the NSCLC progression as a tumor suppressor. Nonetheless, the...
upstream regulatory mechanism of RGMB in NSCLC warrants further investigation.

In this work, we evaluated the expression profile of circRNAs in the NSCLC tissues and found that circ_0001073 expression was markedly down-modulated in NSCLC tissues. We investigated the biological function of circ_0001073 in NSCLC and its relationship with miR-582-3p and RGMB. The results suggested that circ_0001073 suppressed the malignant phenotypes of NSCLC cells via regulating miR-582-3p and RGMB. This work provided new insights into the molecular mechanisms of NSCLC progression.

Materials and Methods

Clinical specimens

The research was endorsed by the Ethics Committee of the First Affiliated Hospital of Baotou Medical College (Baotou, China, 20180043) and written informed consent was obtained from all participating patients. Forty pairs of NSCLC tissues and paracancerous lung tissues were collected during surgery. Then, the tissue samples were frozen at -80°C. None of the subjects underwent radiotherapy or chemotherapy prior to the surgery. The experiments about human tissues were performed according to the Declaration of Helsinki.

Cell culture

LC cell lines [A549 (No. CCL-185), H460 (No. HTB-177), HCC827 (No. CRL-2868), H1299 (No. CRL-5803), and H1975 (No. CRL-5908)] and human bronchial epithelial cells (BEAS-2B, No.CRL-9609) from the American Type Culture Collection (ATCC, Rockville, MD, USA) were used in this study. All cells were maintained in RPMI-1640 medium (Cat No. 11875101, Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Cat No. 10099, Gibco, Carlsbad, CA, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Cat No. 15140163, Gibco, Carlsbad, CA, USA) at 37°C in 5% CO₂.

Cell transfection

The circ_0001073 overexpression plasmid (pcDNA3.1-circ_0001073) was synthesized by GenePharma (Shanghai, China). Empty plasmid (pcDNA3.1-NC) was used as a negative control. siRNAs targeting RGMB (si-RGMB), miR-582-3p mimics, and the corresponding controls (si-NC and miR-con) were designed and synthesized by RiboBio (Guangzhou, China). Moreover, cells were transiently transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) in line with the protocol.

Quantitative real-time polymerase chain reaction

Total RNA was separated from tissues and cells using TRIzol reagent (Cat No. 15596026, Invitrogen, Carlsbad, CA, USA) according to manufacturer’s protocol. Then, 500 ng of total RNA was reversely transcribed into cDNA using Prime Script RT Master Mix (Cat No. RR036A, Takara, Dalian, China). Quantitative real-time polymerase chain reaction (qRT-PCR) was then executed on an ABI7500 system (ABI Biosystems, Foster City, CA, USA) with a Bestar™ qPCR Master Mix (Cat No. #2231, DBI Bioscience, Shanghai, China). Relative expression was calculated using the 2^{-ΔΔCt} method. The primer sequences selected in this research were as follows:

- circ_0001073:
  F: 5’-AAGATGCGCCCTACCTCTCTGT-3’
  R: 5’-CCATAACACCGGTCAACACC-3’
- RGMB:
  F: 5’-GGCCTGGCCACTGACGACATG-3’
  R: 5’-ACTGAACTGACGTCACAGTTGGTA-3’
- GAPDH:
  F: 5’-GACTCATGACCACAGTCCATGC-3’
  R: 5’-AGAGGCGAAGGTGATGTTGCTTG-3’
- miR-582-3p:
  F: 5’-GCACACATTTGAAGAGGACAGAC-3’
  R: 5’-TATTGAAGGGGGTTCTGGTG-3’
- U6:
  F: 5’-CCTAGAAAGCTTTTTTGCGGT-3’
  R: 5’-GAGCTACGAGCTGCCTGACG-3’

(Fig.S1, See Supplementary Online Information at www.celljournal.org).

RNase R assay

Total RNA was separated from A549 and H460 cells. Then, 5 μg of total RNA samples was incubated with 3 U/μg RNase R (Cat No. RNR07250, Epicenter Biotechnologies, Madison, WI, USA) for 20 minutes at 37°C. Subsequently, circ_0001073 expression was determined by qRT-PCR, with GAPDH as a control.

Subcellular distribution experiment

Total RNA from the nuclei and cytoplasm of A549 and H460 cells was extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Cat No. 78835, Thermo Scientific, Waltham, MA, USA). qRT-PCR was applied to determine circ_0001073 expression in the nuclei and cytoplasm, respectively. Besides, U6 and GAPDH served as the nuclear and cytoplasmic controls, respectively.

Cell counting kit-8 experiment

Cell multiplication was detected using CCK-8 (Cat No. C0037, Beyotime, Shanghai, China). A549 and H460 cells were planted in 96-well plates (2×10³ cells per well). At the specific time points (12, 24, 48, 72, and 96 h), 90 μL of serum-free medium and 10 μL of CCK-8
solution were supplemented to each well. The cells were then incubated at 37°C for 2 hours. Using Infinite M200 microplate reader (Tecan, Männedorf, Switzerland), the absorbance of the cells was determined at 450 nm.

**Scratch-healing experiment**

NSCLC cells were planted in 6-well plates and cultured. When the confluence reached 80-90%, the cells were scratched vertically with a pipette tip and rinsed twice with phosphate buffered solution (PBS), and the wound was observed under an inverted microscope, which was recorded as 0 h. After that, the cells were cultured with serum-free medium at 37°C in 5% CO₂, and the wound healing was observed at the same observation point after 24 hours. Scratch healing rate (%) was calculated based on below formula:

\[
\text{Scratch healing rate (\%) = \frac{(0 \text{ h scratch width} - 24 \text{ h scratch width})}{(0 \text{ h scratch width}) \times 100}
\]

**Transwell experiment**

Transwell chambers (Cat No. 3374, Corning, Shanghai, China) were used for migration and invasion assays. Only for invasion assay, we used Matrigel (Cat No. 356234, BD Biosciences, Franklin Lakes, NJ, USA). A549 and H460 cells (5×10⁴ cells) in serum-free medium was added to the top compartment of chamber, and medium containing 10% FBS was supplemented to the bottom of it. After 24 hours culturing at 37°C, 5% CO₂, the upper membrane surface cells were swabbed with a cotton swab, and the migrating or invading cells were fixed with 95% ethanol (Cat No. 400203, Sigma-Aldrich, Louis, MO, USA) and stained with 0.2% crystal violet solution (Cat No. V5265, Sigma-Aldrich, Louis, MO, USA). Finally, the below membrane surface cells was counted under an optical microscope (Nikon, Tokyo, Japan).

**Flow cytometry**

Apoptosis was determined using the Annexin V-FITC apoptosis assay kit (Cat No. BMS500FI-300, Invitrogen, Carlsbad, CA, USA). A549 and H460 cells were trypsinized and rinsed with cold PBS. Subsequently, NSCLC cells (1×10⁶ cells) were resuspended in 100 μL of binding buffer. After 20 minutes of incubation with 5 μL of Propidium iodide (PI) and 5 μL of Annexin V-FITC, apoptotic cells were immediately analyzed by a flow cytometry (BD Biosciences, San Jose, CA, USA).

**Western blot**

Extracting total protein, RIPA lysis buffer (Cat No. P0013C, Beyotime, Shanghai, China) was used, and protein concentration was examined following the manufacturer’s instruction of BCA protein assay kit (Cat No. #5000006, BioRad, Hercules, CA, USA). A total of 20 μg of protein was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to PVDF membranes (Cat No. IPVH00100, Millipore, Bedford, MA, USA). After 1 hour blocking with 5% skim milk, the membranes were incubated with the following primary antibodies overnight at 4°C: anti-RGMB (Cat No. ab96727, Abcam, Shanghai, China, 1:1000) and anti-GADPH (Cat No. ab9485, Abcam, Shanghai, China, 1:1000). Then, the membranes were rinsed with TBST (Cat No. T1085, Solarbio, Beijing, China) and incubated with HRP-coupled goat anti-rabbit secondary antibody (Cat No. ab6721, Abcam, Shanghai, China, 1:2000) for 2 hours at room temperature. The protein bands were examined with the ECL Plus assay kit (Pierce, Rockford, IL, USA), and protein expression was quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA).

**RNA immunoprecipitation experiment**

The RNA binding protein immunoprecipitation (RIP) assay was performed using the Magna RIP Kit (Cat No. 17-700, Millipore, Bedford, MA, USA) according to the manufacturer’s protocols. Then, 1×10⁶ precipitated cells were resuspended in a solution containing RIP lysis buffer, protease and RNAse inhibitors. Also, 100 mL cell lysate was incubated with anti-Ago2 antibody (Cat No. Ab186733, Abcam, Shanghai, China) or immunoglobulin G (IgG; Cat No. MA5-27548, Millipore, Bedford, MA, USA) antibody overnight at 4°C. Immunoprecipitated RNA was isolated using the RNeasy MinElute Cleanup Kit (Cat No. 74204, Qiagen, Shanghai, China). Then reverse transcription was performed by Golden-star™ RT6 cDNA Synthesis Kit (according to the manufacturer’s protocols) (Cat No. TSK302M, TSINGKE, Beijing, China). The immunoprecipitated RNA was detected by qRT-PCR to detect the abundance of circ_0001073 and miR-582-3p.

**Dual-luciferase reporter gene experiment**

Synthesized sequences of circ_0001073, RGMB 3’-UTR and wild-type or mutant miR-582-3p binding sites were cloned into the psi-CHECK2 vector (Promega, Madison, WI, USA). Subsequently, luciferase vector and miR-582-3p mimics (or miR-con) were co-transfected into A549 and H460 cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After 48 hours of transfection, luciferase activity was measured by using dual-luciferase assay system (Promega, Madison, WI, USA).

**Statistical analysis**

Various statistical analyses were performed by SPSS software version 20.0 (IBM Corp., Armonk, NY, USA). Students t test was used to compare the differences between two groups. Comparisons among multiple groups were analyzed by one-way ANOVA followed by a post hoc Tukey test for multiple comparisons. Pearson’s correlation test was used to determine the relationships among circ0001073, miR-582-3p, and RGMB mRNA expressions P<0.05 signified statistical significance.

**Results**

Circ_0001073 was lowly expressed in NSCLC tissues and cells

Analyzing public dataset GSE112214 of microarray data, circ_0001073 expression was down-modulated in NSCLC.
tissues in compare with paracancerous tissues (Fig.1A) that were validated with qRT-PCR results (Fig.1B). In comparison with 16HBE cell line, circ_0001073 was significantly under-expressed in the NSCLC cell lines (Fig.1C). Furthermore, bioinformatics analysis uncovered that circ_0001073 was an exonic circRNA consisting of three exons (exons 3, 4, and 5) of ACVR2A gene (Fig.1D) (18). RNase R assay indicated that circ_0001073 was resistant to RNase R digestion, while GAPDH mRNA was sensitive (Fig.1E). Additionally, subcellular distribution analysis signified that circ_0001073 was predominantly located in the cytoplasm of NSCLC cells (Fig.1F). We observed that the NSCLC patients with higher expression level of circ_0001073 had a longer survival time compared with the patients with lower expression level of circ_0001073 (Fig.1G).

Circ_0001073 overexpression restrained the multiplication, migration, and invasion of NSCLC cells and enhanced the apoptosis

To elaborate on the effects of circ_0001073 on the proliferation, migration and invasion of NSCLC cells, circ_0001073 overexpression plasmids were selected to be transfected into A549 and H460 cells with the lowest circ_0001073 expression. The result of overexpression efficiency was illustrated in Figure 2A that has achieved by 48 hours after the transfection qRT-PCR. The multiplication of cells was examined by CCK-8 experiment. The results indicated that circ_0001073 overexpression significantly inhibited the multiplication of A549 and H460 cells of NC group (Fig.2B). The data of scratch-healing experiments and Transwell experiments demonstrated that the cell migration and invasion in the circ_0001073 overexpression group were significantly decreased compared with NC group (Fig.2C-H). Additionally, flow cytometry analysis manifested that circ_0001073 overexpression significantly promoted the apoptosis of NSCLC cells (Fig.2I-J).
**Circ_0001073** acted as a molecular sponge for *miR-582-3p* in NSCLC cells

Also, circRNAs mainly located in the cytoplasm, which has suggested that usually function as sponges for miRNAs (19). To pinpoint the underlying mechanism of circ_0001073 in the NSCLC, target miRNAs of circ_0001073 were predicted by bioinformatic analysis tools (the result is available upon request). A complementary binding sequence was discovered between circ_0001073 and *miR-582-3p* (Fig.3A). To validate the targeting relationship between them, dual-luciferase reporter gene experiment was conducted. In the A549 and H460 cells, data implied that high expression of *miR-582-3p* represses the luciferase activity of circ_0001073-WT, while had not any effect on circ_0001073-MUT (Fig.3B). RIP assay showed that circ_0001073 and *miR-582-3p* were significantly enriched in the Ago2 group of A549 and H460 cells (Fig.3C). Moreover, circ_0001073 overexpression significantly decreased *miR-582-3p* expression in A549 and H460 cells, indicating that *miR-582-3p* expression was regulated by circ_0001073 (Fig.3D). We observed that *miR-582-3p* was significantly higher expressed in NSCLC tissues and cells compared with paracancerous tissues or 16HBE cells (Fig.3E, F). Correlation analysis showed a negative correlation between *miR-582-3p* expression and circ_0001073 expressions in the NSCLC tissues (Fig.3G). The above data confirmed that circ_0001073 could sponge *miR-582-3p* and inhibit its expression.

**RGMB** was a downstream target gene of **miR-582-3p**

Using the StarBase database, potential target genes of *miR-582-3p* were predicted that may elucidate the downstream mechanism of the circ_0001073/miR-582-3p axis in NSCLC (the result is available on request). A complementary binding sequence was discovered between *miR-582-3p* and *RGMB*’s UTR, that was shown in Figure 4A. Dual-luciferase reporter experiments confirmed that *miR-582-3p* overexpression remarkably inhibits the luciferase activity of RGMB-WT in A549 and H460 cells whereas it exerts no significant effect on the luciferase activity of RGMB-MUT (Fig.4B). Moreover, qRT-PCR analysis showed that *miR-582-3p* expression was remarkably up-regulated in A549 and H460 cells transfected with *miR-582-3p* mimics (Fig.4C). The data of qRT-PCR and Western blot reveals that *miR-582-3p* overexpression remarkably inhibits RGMB mRNA and protein expression in NSCLC cells relative to the miR-con group (Fig.4D, E). Additionally, RGMB mRNA expression was remarkably down-modulated in NSCLC tissues and cells (Fig.4F, G). Correlation analysis showed that RGMB mRNA expression was negatively correlated with *miR-582-3p* expression and positively associated with circ_0001073 expression in NSCLC tissues (Fig.4H, I). Furthermore, RGMB mRNA and protein expressions were remarkably down-regulated in the si-RGMB transfected cells. Since si-RGMB#1 had the highest knockdown efficiency (Fig.4J, K), therefore, we selected it for our subsequent experiments.

![Fig.3: MiR-582-3p was the target of circ_0001073. A. Bioinformatics analysis predicted the binding sequence between miR-582-3p and circ_0001073. B. Dual-luciferase reporter gene experiments were used to verify the binding relationship between miR-582-3p and circ_0001073. C. The enrichment of circ_0001073 and miR-582-3p in Ago2 or IgG immunoprecipitate was determined using the RIP method. D. MiR-582-3p expression in the circ_0001073 overexpression plasmid transfected cells was detected by qRT-PCR analysis. E, F. Using qRT-PCR, MiR-582-3p expression was detected in the NSCLC tissues and cells. G. Pearson’s correlation analysis was employed to analyze the correlation between miR-582-3p expression and circ_0001073 expression in the NSCLC tissues. All experiments were performed in triplicate.*: P<0.05, NC: Negative control, NSCLC; Non-small cell lung cancer, and qRT-PCR; Quantitative real-time polymerase chain reaction.](image-url)

**Circ_0001073** up-regulated RGMB expression by targeting miR-582-3p to inhibit NSCLC progression

qRT-PCR and Western blot analysis showed that overexpression circ_0001073 remarkably increased RGMB expression, while *miR-582-3p* mimics or si-RGMB#1 transfection reduced this phenomenon (Fig.5A, B). We observed that *miR-582-3p* mimics or si-RGMB#1 weakened the effects of circ_0001073 overexpression on the multiplication, migration, and invasion of A549 and H460 cells (Fig.5C-F). Furthermore, flow cytometry analysis revealed that the promotion of apoptosis by circ_0001073 overexpression could be attenuated by *miR-582-3p* mimics or si-RGMB#1 (Fig.5G). The above results indicated that the *miR-582-3p/RGMB* axis is vital for maintaining the function of circ_0001073 in NSCLC cells.
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**Fig. 4:** RGMB was a downstream target of miR-582-3p in NSCLC cells.  
(A) Bioinformatics analysis predicted the binding sequence between miR-582-3p and RGMB 3’UTR.  
(B) The binding relationship between miR-582-3p and RGMB was detected using the dual-luciferase reporter gene assay.  
(C) qRT-PCR analysis was used to detect miR-582-3p expression in A549 and H460 cells after the transfection with miR-582-3p mimics.  
(D, E) The RGMB expressions (mRNA and protein) in A549 and H460 cells transfected with miR-582-3p mimics were detected using qRT-PCR and Western blot.  
(F, G) Using qRT-PCR, mRNA expression of RGMB was detected in NSCLC tissues and cells.  
(H, I) Pearson’s correlation analysis was used to analyze the correlation between RGMB mRNA and miR-582-3p or circ_0001073 expression in NSCLC tissues.  
(J, K) RGMB expression (mRNA and protein) in si-RGMB transfected cells (A549 and H460) was detected by qRT-PCR analysis and Western blot. All experiments were performed in triplicate. *; P<0.05, NSCLC; Non-small cell lung cancer, and qRT-PCR; Quantitative real-time polymerase chain reaction.

**Fig. 5:** circ_0001073 modulated NSCLC progression via the miR-582-3p/RGMB axis.  
(A, B) RGMB mRNA and protein expression was determined by qRT-PCR and Western blot.  
(C) Cell multiplication was assessed using the CCK-8 method.  
(D) Cell migration was assessed by wound-healing experiment.  
(E, F) Transwell experiments were used to detect cell migration and invasion.  
(G) Flow cytometry was used to detect the apoptosis of NSCLC cells. All experiments were performed in triplicate. *; P<0.05, NC; Negative control, NSCLC; Non-small cell lung cancer, and qRT-PCR; Quantitative real-time polymerase chain reaction.

**Discussion**

CircRNAs exhibit unique expression pattern and specific function in tumor progression (20, 21). Several lines of evidences have indicated that, aberrant circRNA expression is associated with the tumorigenesis and progression of NSCLC. For instance, circ_0001073...
expression is up-regulated in the NSCLC tissues; circ_0000376 overexpression promotes the multiplication and metastasis of the NSCLC cells and enhances their chemoresistance (22). In contrast, circ_0002483 is under-expressed in NSCLC tissues and cell lines, which inhibits NSCLC progression by targeting miR-182-5p and enhances the sensitivity of cancer cells to paclitaxel (23). Some studies have shown that circ_0001073 is under-expressed in bladder cancer and breast cancer (24, 25). Circ_0001073 overexpression restraints the multiplication and metastasis of bladder cancer cells (24). Moreover, the down-modulation of circ_0001073 expression is linked to the unfavorable breast cancer prognosis in these patients, and circ_0001073 overexpression inhibits breast cancer cell multiplication and induces apoptosis (25). Our result confirmed that circ_0001073 expression was markedly down-modulated in NSCLC tissues and cells and circ_0001073 overexpression impeded the multiplication, migration, and invasion of NSCLC cells and induced apoptosis. Our results suggested that circ_0001073 was a tumor suppressor in NSCLC.

CircRNAs can participate in regulating cancer development by functioning as miRNA sponges to modulate miRNA expression (26). For instance, circ_0026134 enhances the multiplication and invasion of NSCLC cells by sponging miR-1256 and miR-1287 (27). Circ_ZNF124 activates the JAK2/STAT3 signaling pathway by targeting miR-337-3p, thereby promotes NSCLC development (28). In this work, circ_0001073 was confirmed to act as a molecular sponge for miR-582-3p and negatively regulator of miR-582-3p expression in the NSCLC cells. The biological functions of miR-582-3p are investigated in many cancers. Huang et al. (12), reported that, miR-582-3p and miR-582-5p inhibit bone metastasis of prostate cancer by impairing TGF-β signaling; also, Li et al. (13), observed that miR-582-3p negatively modulates the multiplication and cell cycle progression of acute myeloid leukemia cells by targeting cyclin B2. Importantly, miR-582-3p exerts a carcinogenic effect in NSCLC: it inhibits the apoptosis of A549, NCI-H1703, and NCI-H1975 cells, and enhances their stem cell properties (14). Consistently, the present study confirmed that miR-582-3p was remarkably overexpressed in NSCLC tissues and cell lines. In addition, miR-582-3p markedly counteracted the effects of circ_0001073 multiplications, migration, invasion, and apoptosis of the NSCLC cell. The above data implied that circ_0001073 could exert tumor-suppressive effects in NSCLC progression by targeting miR-582-3p.

RGMB, a member of RGM family, is a regulator in the regeneration and remodeling of axons and synapses and a co-receptor for bone morphogenetic protein (BMP) (29-31). Also, RGM family included RGMA, RGMB, and RGMC (29). RGMB can directly interact with the BMP receptors of BMP-2 and BMP-4, thereby augmenting the binding to ligands (31). Involvement in the BMP signaling pathway, RGMB is implicated in cancer initiation and development (32). Reportedly, RGMB expression is up-regulated in the colorectal cancer tissues, that subsequently, inhibits oxaliplatin-induced phosphorylation of JNK and p38 MAPK and reduces oxaliplatin-induced apoptosis (33). In squamous cell carcinoma of the head and neck, RGMB, targeted by miR-93-5p, participates in regulating the migration and invasion of tumor cells (34). Importantly, RGMB inhibits NSCLC progression via regulating Smad1/5/8 pathway (16). Notably, the present work revealed that RGMB expression is down-modulated in the NSCLC tissues and cells. Additionally, RGMB was confirmed to be a downstream target gene of miR-582-3p, and RGMB expression was negatively correlated with miR-582-3p expression and positively associated with circ_0001073 expression in NSCLC tissues. What’s more, transfection of miR-582-3p mimics or si-RGMB remarkably reversed the suppressive effects of circ_0001073 on NSCLC cell multiplication, migration, and invasion and apoptosis promotion. These demonstrations suggest that the circ_0001073/miR-582-3p/RGMB axis was present in the NSCLC.

Collectively, this study found that the expression of circ_0001073 was down-regulated in the NSCLC tissues and cells that participates in regulating the proliferation, migration, invasion, and apoptosis of NSCLC cells by modulating miR-582-3p/RGMB axis. To our knowledge, this is the first report to reveal the interactions among circ_0001073, miR-582-3p and RGMB in the NSCLC. However, our demonstrations are only based on in vitro assays, and the conclusions should be validated by in vivo research in the following studies. In the future, studies will be concentrated on, the identification of other circ_0001073 downstream miRNAs.

Conclusion
This study elucidates that circ_0001073 up-regulates RGMB expression by targeting miR-582-3p that associated with inhibiting the proliferation, migration, and invasion of NSCLC cells and inducing cell apoptosis. This study reveals a new molecular mechanism in the progression of NSCLC and provides new insights into the treatment of NSCLC.

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Authors’ Contributions
C.W.; Conceive and experiments design. X.J., M.R.,
Yo.F., Yu.F.; Experiments performing. Yo.F., Yu.F.; Data analysis. X.J., M.R., Yo.F., Yu.F.; Manuscript writing. All authors read and approved the final manuscript.

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