CircABCB10 promotes nonsmall cell lung cancer cell proliferation and migration by regulating the miR-1252/FOXR2 axis

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Abstract
Circular RNA (circRNA) is a key regulator in the development and progression of human cancers. Previous studies confirmed circRNA-0008717 (circABCB10) as an oncogene in osteosarcoma, but the regulatory effect of circABCB10 in nonsmall cell lung cancer (NSCLC) is still unclear. In the current study, we examined the expression of circABCB10 in different NSCLC cell lines. Bioinformatics analysis, Cell Counting Kit-8 assays, Transwell migration, fluorescein reporting experiments, and xenografts in mice were used to detect the effect of circABCB10 on NSCLC cell proliferation and migration in vitro and tumor growth in vivo. The results showed that the expression of circABCB10 in NSCLC cell lines was increased. Downregulation of circABCB10 suppressed NSCLC cell proliferation and migration by promoting microRNA miR-1252 expression and suppressing Forkhead box 2 (FOXR2). Fluorescein reporting experiments confirmed that circABCB10 expression increased FOXR2 levels by sponging miR-1252, and in vivo experiments found that knockdown of circABCB10 decreased tumor growth. These data suggested that circABCB10 acted as a tumor promoter through a novel miR-1252/FOXR2 axis, providing potential biomarkers and therapeutic targets for the management of NSCLC.

Keywords
circABCB10, FOXR2, miR-1252, nonsmall cell lung cancer, proliferation and migration

1 | INTRODUCTION

Lung cancer is a leading cause of death and is becoming more prevalent worldwide.1 The most frequent type of lung cancer is nonsmall cell lung cancer (NSCLC, approximately 85% of all lung cancers). Low-income and middle-income countries now account for more than 50% of lung cancer deaths each year. Although treatments are improving, the 5-year survival rate is still only 17% for NSCLC.2,3 Therefore, a better understanding of the molecular mechanisms associated with NSCLC pathogenesis is critical to the development of effective diagnostic and therapeutic approaches, which may lead to the identification of novel biomarkers and treatment targets for NSCLC and possibly to the development of personalized therapies for individual NSCLC patients in the future.4

As a noncoding RNA, circular RNAs (circRNAs) have become an important research topic in recent years.5

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However, the biological functions of circRNAs in human diseases are still unclear, especially in cancer pathogenesis. Recently, studies have found that several circRNAs possess microRNA (miRNA) binding sites and can regulate miRNA downstream targets by sponging and arresting the activity of miRNAs.\textsuperscript{6-8} One study found that circRNA-0008717 (circABCB10) was upregulated in osteosarcoma, suggesting its use as a prognostic biomarker for osteosarcoma patients.\textsuperscript{9} The circABCB10 is cyclized from part of the ABCCB10 gene, but its role in NSCLC remains unclear.

Therefore, in the current study, we compared circABCB10 expression between normal lung epithelial cells and lung cancer cell lines. The effects of circABCB10 expression on cell migration and proliferation of lung cancer cell lines were also investigated. Bioinformatics analysis and fluorescence reporting experiments suggested an interaction between circABCB10, miR-1252, and Forkhead box 2 (FOXR2). We found that circABCB10 played an oncogenic role in NSCLC by sponging miR-1252 to increase FOXR2 synthase expression and consequently promote NSCLC progression. Therefore, circABCB10 may serve as a useful biomarker for prognosis predictions and as a potential therapeutic target for NSCLC patients.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All animal experiments were approved and performed according to the guidelines of the Ethics Committee of Zhongshan Hospital of Fudan University, Shanghai, China. All surgical procedures were performed under anesthesia, and every effort was made to minimize suffering.

2.2 | Cell culture and transfection

Breast cancer cell lines (A549, H1975, PC9, H1299, and H1650) and normal human lung epithelial cells (BEAS-2B) were purchased from the American Type Culture Collection (Manassas, VA) and cultured in Roswell Park Memorial Institute Medium 1640 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) with 100 IU/mL of penicillin and 100 μg/mL of streptomycin at 37°C in 5% CO\textsubscript{2}.

Small interfering RNA (siRNA) against hsa_circ_0008717 (circABCB10; 5′-TAGAAGACCATGGGGATGTCAAGAG-CATCCCCCATGGCTTTATTTT-3′), miR-1252 mimic (5′-AGAAGGAAAAGAUUCAUUUA-3′), and miR-1252 inhibitor (synthesized by GenePharma, Shanghai, China) were transfected into lung cancer cells using Lipofectamine 2000 Reagent (Invitrogen).

For circABCB10 overexpression, circABCB10 overexpression vector was constructed into pcDNA3.0 and transfected into BEAS-2B cell with the help with Lipofectamine 2000.

2.3 | Bioinformatics analysis

The hsa_circ_0008717 (circABCB10)-miRNA target gene prediction was performed using the https://circinteractome.nia.nih.gov/ website. The miR-1252–mRNA target gene prediction was performed using the http://www.targetscan.org/vert_71/ website.

2.4 | Cell Counting Kit-8 assay

A549 and PC9 cells transfected with or without siRNA against circABCB10 and a FOXR2 overexpression vector were seeded into 96-well plates at 5 × 10\textsuperscript{3} cells/well and incubated for increasing durations (0, 12, 24, 48, and 72 hours) before adding 10 μL of Cell Counting Kit-8 (CCK8) solution per well for 1 hour. Absorbance (450 nm) was measured using a Synergy microplate reader (BioTek, Winooski, VT).

2.5 | Transwell migration assays

Cell migration was analyzed using Transwell chambers (Corning, New York, NY) according to the manufacturer’s protocol. After incubation for 24 hours, cells on the upper surfaces of the Transwell chambers were removed with cotton swabs, and the cells located on the lower surfaces were fixed in methanol for 10 minutes, followed by staining with crystal violet. The stained cells were photographed and counted in five randomly selected fields.

2.6 | Luciferase reporter assay

For luciferase reporter assays, wild-type (wt) and mutant (mut) promoters of circABCB10 and wt and mut sequences from the 3′-untranslated region (3′-UTR) of FOXR2 were synthesized and inserted into pGL3-basic vectors (GeneCreat, Wuhan, China). The pGL3-basic-circABCB10-wt/mut vectors, pGL3-basic-FOXR2-wt/mut vectors, and pcDNA3.1-c-Myb vectors were cotransfected with pRL-TK into HEK293T cells using Lipofectamine 2000. For circABCB10 and miR-1252 luciferase reporter assays, circABCB10 and FOXR2 3′-UTR sequences containing wt or mutated miR-1252 binding sites were synthesized and inserted into the pmirGLO luciferase vector (Gene-Creat) and then cotransfected with miR-1252 mimics into HEK293T cells using Lipofectamine 2000. Cells were harvested 48 hours after transfection, and luciferase activity was detected with the dual-luciferase reporter assay system.
Relative luciferase activity was normalized to the Renilla luciferase internal control.

2.7 | Xenografts in mice

A549 cells were stably infected with or without circABCB10 silencing vectors. A total of 5 × 10⁶ viable cells were injected into the right flanks of nude mice as described previously. Tumor sizes were measured using a vernier caliper every 5 days from the fifth day after injection, and tumor volume was calculated using the following formula: volume = 1/2 × length × width². At 30 days after implantation, mice were euthanized for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blot analyses.

2.8 | Quantitative reverse transcription-polymerase chain reaction

RNA was extracted from tissues or cultured cells with TRIzol reagent according to the manufacturer’s protocol (Life Technologies, Scotland, UK). Reverse transcription of 200 ng of total RNA was performed in a final volume of 10 μL using random primers and standard conditions with the Prime Script RT Master Mix (Applied Biosystems, Foster City, CA). qRT-PCR was performed using the SYBR Select Master Mix (Applied Biosystems) with 0.5 μL of complementary DNA (cDNA) on the ABI7300 system (Applied Biosystems) according to the manufacturer’s instructions. We used glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or U6 as internal controls. Relative transcription alterations were evaluated using the 2−ΔΔCt method as previously described.

2.9 | Western blot analysis

Protein was extracted from cells or tissues using RIPA lysis buffer (Applied Biosystems) and subjected to Western blot analysis as described previously. Anti-FOXR2 and anti-GAPDH antibodies were supplied by Cell Signaling Technology (Beverly, MA). All antibodies were used according to the manufacturer’s protocol, and immunoreactivity was visualized using a chemiluminescence detection kit (ECL Western Blotting Substrate; Dongguan Biotech, Shandong, China).

2.10 | Statistical analysis

Data were analyzed using the SPSS software package (IBM, Armonk, NY). Statistical significance was determined with the Student t test or analysis of variance, and P < 0.05 was considered statistically significant.
that miR-1252 was a potential target of circABCB10. To prove this prediction, we conducted luciferase reporter assays (Figure 3A) and showed that miR-1252 overexpression significantly inhibited luciferase activity of the circABCB10-wt reporter in both A549 and PC9 cells (Figure 3B and 3C), indicating a direct interaction between circABCB10 and miR-1252. The qRT-PCR assays showed that knockdown of circABCB10 promoted miR-1252 expression in both A549 and PC9 cells (Figure 3D), suggesting that circABCB10 could sponge miR-1252.

### 3.4 | CircABCB10 promoted FOXR2 expression by sponging miR-1252

Our bioinformatics analysis further suggested that miR-1252 could interact with the 3′-UTR of FOXR2 (Figure 4A). In confirmation, luciferase reporter assays showed that
**FIGURE 2** Overexpression of circABCB10 promotes BEAS-2B proliferation and migration. A, Relative expression levels of circABCB10 were measured by qRT-PCR in BEAS-2B after transfected with circABCB10 overexpression vector or negative control (NC). B, CCK8 assays were used to detect the proliferation of BEAS-2B cells. C, Cell migration of BEAS-2B cells was measured by Transwell assay. Each experiment was repeated at least three times; *P < 0.05, **P < 0.01, and ***P < 0.001. CCK8, Cell Counting Kit-8; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

**FIGURE 3** The miR-1252 was the target of circABCB10. A, Predicted binding sites of miR-1252 and circABCB10. B, C, Luciferase assay of A549 and PC9 cells cotransfected with miR-1252 mimics and a luciferase reporter containing circABCB10-wt or mutant constructs (circABCB10-mut). D, CircABCB10 knockdown promoted miR-1252 expression in both A549 and PC9 cells. Each experiment was repeated at least three times; ***P < 0.001. Mut, mutant; NC, negative control; Wt, wild type.
miR-1252 overexpression significantly inhibited luciferase activity of the FOXR2 3′-UTR-wt reporter in both A549 and PC9 cells (Figure 4B and 4C), indicating a direct interaction between the 3′-UTR of FOXR2 and miR-1252. The qRT-PCR and Western blot analysis confirmed that miR-1252 overexpression suppressed the expression of FOXR2 messenger RNA (mRNA) and protein, respectively (Figure 4D and 4E) and demonstrated that circABCB10 knockdown also decreased FOXR2 mRNA and protein expression in both A549 and PC9 cells (Figure 4F and 4G).

The qRT-PCR experiments showed that circABCB10 silencing significantly suppressed FOXR2 expression, whereas inhibition of miR-1252 rescued FOXR2 levels in both A549 and PC9 cells (Figure 4H). CCK8 analysis found that miR-1252 inhibitor treatment reversed the inhibition of cell proliferation induced by circABCB10 silencing in both A549 and PC9 cells (Figure 4I and 4J). Transwell analysis also found that downregulation of miR-1252 rescued the loss of migration induced by circABCB10 silencing in both A549 and PC9 cells (Figure 4K and 4J). These results suggested that circABCB10 promoted FOXR2 expression by sponging miR-1252, which inhibited lung cancer cell proliferation and migration.
Finally, we sought to analyze the effects of circABCB10 on tumor growth in vivo. We performed a xenograft experiment by injecting A549 cells into nude recipient mice. Tumor size was measured and calculated every 5 days. The results show that circABCB10 knockdown significantly inhibited tumor growth (Figure 5A). Furthermore, after 30 days postinjection, we measured tumor weights and found that circABCB10 silencing led to smaller tumors (Figure 5B). The qRT-PCR detection found that the expression of miR-1252 was increased more in tumor tissues from the circABCB10-silenced group than that in the NC group (Figure 5C). Western blot analyses revealed that FOXR2 expression in tumor tissues from the circABCB10-silenced group was decreased when compared with the NC group (Figure 5D).

3.5 Knockdown circABCB10 inhibited lung cancer growth in vivo

Finally, we sought to analyze the effects of circABCB10 on tumor growth in vivo. We performed a xenograft experiment by injecting A549 cells into nude recipient mice. Tumor size was measured and calculated every 5 days. The results show that circABCB10 knockdown significantly inhibited tumor growth (Figure 5A). Furthermore, after 30 days postinjection, we measured tumor weights and found that circABCB10 silencing led to smaller tumors (Figure 5B). The qRT-PCR detection found that the expression of miR-1252 was increased more in tumor tissues from the circABCB10-silenced group than that in the NC group (Figure 5C). Western blot analyses revealed that FOXR2 expression in tumor tissues from the circABCB10-silenced group was decreased when compared with the NC group (Figure 5D).

4 DISCUSSION

Lung cancer is the leading cause of cancer deaths worldwide. Previous studies have found that miRNAs and long noncoding RNAs play an important role in lung cancer development and progression. Whether circRNAs exert similar roles in tumorigenesis remains unclear. In the current study, we found that circABCB10 expression was increased in lung cancer cells. Knockdown of circABCB10 suppressed proliferation and migration of both A549 and PC9 cells. Bioinformatics analysis found that miR-1252 was the target of circABCB10, which was confirmed by fluorescein reporting experiments. The results also found that circABCB10 silencing promoted miR-1252 expression, suggesting that circABCB10 may act as an miR-1252 sponge. Further bioinformatics analysis found that FoxR2 was the target of miR-1252. Fluorescein reporting experiments confirmed that miR-1252 interacted with the 3′-UTR of FoxR2 and suppressed FoxR2 expression posttranscriptionally.

Human Fox genes belong to the family of winged/forkhead transcription factors, including at least 43 members from FoxA1 to FoxQ1. FoxR2 is a more recently discovered member of the Fox transcription factor family, first identified in 2004. An increasing number of studies have found that FoxR2 plays an important role in the development of many human tumors, including glioma, prostate cancer, hepatocellular carcinoma, and even NSCLC. Knockdown of FOXR2 suppressed...
tumorigenesis, growth, and metastasis. In the current study, we found that overexpression of miR-1252 suppressed FOXR2 expression. Silencing circABCB10 suppressed FOXR2 expression, suggesting that circABCB10 expression promoted FOXR2 by sponging miR-1252. Silencing of miR-1252 reversed circABCB10 knockdown-induced proliferation and inhibited migration of lung cancer cells by promoting FOXR2 expression, which suggested that circABCB10 promoted FOXR2 expression, suggesting that circABCB10 may be a potential target for NSCLC therapy.

In summary, our study has for the first time to our knowledge investigated the role of circABCB10 and identified it as an oncogene in NSCLC. Our findings demonstrated that circABCB10 contributed to NSCLC progression by enhancing FOXR2 expression via sponging of miR-1252, suggesting that circABCB10 may be a potential target for NSCLC therapy.

CONFLICTS OF INTEREST
The authors declare that there are no conflicts of interest.

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