

miRNA-99b-5p targets FZD8 to inhibit non-small cell lung cancer proliferation, migration and invasion

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Background: miRNAs were found to play crucial roles in regulating cellular behaviors. The aim of this study was to investigate the biological function of miRNA-99b-5p (miR-99b-5p) in non-small cell lung cancer (NSCLC).

Materials and methods: miR-99b-5p expression level in NSCLC cell lines was detected by quantitative real-time PCR (qRT-PCR). Cell proliferation, migration and invasion were examined by cell counting kit-8 (CCK-8) assay, wound-healing assay and Transwell invasion assay, respectively. Dual-luciferase activity reporter assay and Western blot assay were conducted to validate the target of miR-99b-5p.

Results: The expression of miR-99b-5p was decreased in NSCLC cell lines compared with normal cell line. Overexpression of miR-99b-5p inhibits cell proliferation, migration and invasion in vitro. FZD8 was validated as a direct target of miR-99b-5p. Overexpression of FZD8 partially abolished the effects of miR-99b-5p mimic on NSCLC cell behaviors.

Conclusion: Collectively, our results demonstrated that miR-99b-5p inhibits NSCLC cell proliferation, migration and invasion through targeting FZD8. This newly identified miR-99b-5p/FZD8 axis provided novel insights into the mechanisms underlying NSCLC progression.

Keywords: miR-99b-5p, FZD8, non-small cell lung cancer, migration, invasion

Introduction

Lung cancer is one of the most common causes of cancer-related death worldwide.¹ There are two major subgroups of lung cancer: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC).² NSCLC alone represents 85% of all lung cancer cases.³ Five-year overall survival of NSCLC patients remains undesirable even though there are significant improvements in treatment measures.⁴ Investigation of biomarkers closely associated with NSCLC tumorigenesis will contribute to control NSCLC progression.

miRNAs are noncoding RNAs that were capable to regulate gene expression through 3'-UTR binding and thus affect cell behaviors, disease initiation and progression.⁵ miRNAs were reported to have dual roles in the tumorigenesis of human cancers: either tumor-suppressive miRNA or oncogenic miRNA.⁶ Oncogenic miRNAs refer to miRNAs frequently upregulated, while tumor-suppressive miRNAs represent miRNAs often downregulated in human cancers.⁶

Mounting studies have been carried out to investigate the abnormal expression of miRNA in NSCLC tissues and cell lines.⁷⁻¹⁰ Guan et al⁷ reviewed previously published literatures concerning miRNA expression profile in NSCLC tissues and identified 184 differentially expressed miRNAs. Wang et al⁸ investigated miRNA profiles in seven NSCLC datasets and identified 11 key miRNAs in NSCLC carcinogenesis.

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These identified miRNAs might be profitable to diagnosis or prognostic prediction of NSCLC.⁸ Besides that, Chen et al⁹ found that miR-148a expression was downregulated in NSCLC tissues compared with normal lung tissues. They also found that low miR-148a expression was correlated with advanced tumor grade, lymph node metastasis and a higher risk of tumor-related death.⁹ Also, miR-148a overexpression was shown to inhibit NSCLC cell migration and invasion through targeting Wnt family member 1.⁹ Wang et al¹⁰ investigated that miR-142-5p overexpression decreased phosphatidylinositol-4,5-bisphosphate 3-kinase and catalytic subunit alpha expression to inhibit NSCLC proliferation in vitro and in vivo. These results indicated the importance of miRNAs in NSCLC carcinogenesis.

FZD8 expression was revealed to be significantly overexpressed in primary lung cancer tissue samples and cell lines compared with their adjacent normal tissues and normal cell line.¹¹ Importantly, knockdown of FZD8 expression in NSCLC cells inhibited proliferation in vitro and suppressed tumor growth in vivo.¹¹ Another study conducted by Jin et al¹² showed that FZD8 was a direct target of miR-375 in SCLC cell line. These results indicated the importance of FZD8 in lung cancer. miRNA-99b-5p (miR-99b-5p) was reported to function as a tumor suppressor in multiple human cancers including gastric cancer, clear-cell renal cell carcinoma and colorectal cancer.^{13–15} However, the biological roles of miR-99b-5p in NSCLC remain unclear. In this study, we analyzed miR-99b-5p expression in NSCLC cell lines and normal cell line. Furthermore, we analyzed the effects of miR-99b-5p expression on NSCLC cell proliferation, migration and invasion. Moreover, the effects of miR-99b-5p on the overall survival of NSCLC patients were analyzed at Kaplan–Meier plotter website. Luciferase activity reporter assay and Western blot assay were conducted to validate whether FZD8 was a functional target of miR-99b-5p.

Materials and methods

Cell culture and cell transfection

NSCLC cell lines (A549 and NCI-H292), SCLC cell line (NCI-H69), human bronchial epithelial cells (16-HBE) and human embryonic kidney-derived cell line (HEK293T) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cell lines were maintained at DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Thermo Fisher Scientific), streptomycin (100 mg/mL) and penicillin (100 U/mL) in a 37°C humidified incubator containing 5% of CO₂.

Cells were transfected with 100 nM of miR-99b-5p mimic or negative control (NC-miRNA) obtained from GenePharma (Shanghai, People's Republic of China) and 4 µg FZD8 overexpression construct (pcDNA3.1-FZD8) or pcDNA3.1 (GenScript, Nanjing, People's Republic of China) using Lipofectamine 2000 reagent (Thermo Fisher Scientific) as per the manufacturer's instructions.

Cell proliferation assay

Cell counting kit-8 (CCK-8) assay was performed to measure the rate of cellular proliferation. Cells were seeded at the 96-well plate at a density of 3,000 cells/well and incubated for 0, 24, 48 or 72 h. Then, CCK-8 reagent was added to each well and incubated at 37°C for additional 3 h, after which the optical density was measured at 450 nm using MRX II microplate reader (Dynex Technologies, Chantilly, VA, USA).

Wound-healing assay

Cells were seeded in six-well plate and incubated until approximately 100% confluence. Then, a wound at cell surface was created using pipette tip, after which cells were washed to remove cell debris. Cell images were captured at 0 or 48 h after wound creation to assess cellular migration rate.

Transwell invasion assay

Cellular invasion ability was analyzed using 24-well Transwell chamber with a Matrigel (BD Biosciences, San Jose, CA, USA) pre-coated 8 µm corning filter (Corning Inc., New York, NY, USA). 1×10⁴ cells in serum-free DMEM were plated to the upper chamber of Transwell plate. The lower chamber was filled with DMEM supplemented with 10% FBS. Forty-eight hours after incubation, noninvasive cells were removed using cotton, while the invaded cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Invasive cell numbers were counted from five randomly selected fields using microscope.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cultured cells using Trizol reagent (Beyotime, Haimen, People's Republic of China) following the manufacturer's instructions. cDNA libraries were synthesized using Reverse Transcription Kit (Takara, Dalian, People's Republic of China). qRT-PCR was conducted at ABI 7500 PCR system (Bio-Rad Laboratories Inc., Hercules, CA, USA) using SYBR Premix Ex Taq (Takara). miR-99b-5p expression level was analyzed using comparative

threshold cycle method with U6 snRNA as internal control. The primers used were as follows: miR-99b-5p: forward: 5'-CACCCGTAGAACCGACCTT-3', reverse: 5'-GTCGTATCCAGTGC GTGTCGTGGAGTCGGCAATTG CACTGGATACGACCGCAAGG-3'; U6 snRNA: forward: 5'-GCTTCGGCAGCACATATACTAAAAT-3', reverse: 5'-CGCTTCACGAATTTGCGTGCAT-3'.

Western blot

RIPA lysis buffer containing protease inhibitors and phosphatase inhibitors (Beyotime) was employed to extract proteins from cultured cells. Equal amounts of protein lysates were separated using 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Beyotime). Membranes were blocked with 5% skim milk, then incubated with primary antibodies (anti-FZD8: ab155093; anti-GAPDH: ab181602; both from Abcam, Cambridge, MA, USA) for overnight at 4°C. After washing with TBST, the membranes were incubated with secondary antibody (ab6721; Abcam) in room temperature at 1 h. Bands were visualized using BeyoECL kit (Beyotime) and ImageJ 1.42 software (NIH, Bethesda, MD, USA).

Bioinformatical analysis and luciferase activity reporter assay

TargetScan (<http://www.targetscan.org>) was used to predict the potential targets of miR-99b-5p. Wild-type (wt) 3'-UTR of FZD8 was inserted into pGL3 vector (Promega Corporation, Fitchburg, WI, USA) and named as FZD8-wt. FZD8-mt was constructed based on FZD8-wt using site-directed

mutagenesis kit (Takara). Cells were co-transfected with FZD8-wt or FZD8-mt and miR-99b-5p mimic or NC-miRNA using Lipofectamine 2000. Forty-eight hours after transfection, relative luciferase activity was measured using a dual-luciferase reporter assay kit (Promega Corporation).

Kaplan–Meier survival analysis

Kaplan–Meier plotter (www.kmplot.com) was used to investigate the effect of miR-99b-5p expression on overall survival of NSCLC patients.¹⁶ Cutoff value was auto selected in the algorithm. Log-rank test was used to calculate the difference between low or high expression groups.

Statistical analyses

Data were presented as mean \pm SD. Differences between groups or among three or above groups were evaluated using the Student's *t*-test or ANOVA and Tukey's post hoc test, respectively. GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) was used for all data analyses. $P < 0.05$ was regarded as statistical significance.

Results

miR-99b-5p expression was reduced in NSCLC

We found that miR-99b-5p expression was significantly reduced in NSCLC cell lines investigated compared with 16-HBE or HEK293T cell line ($P < 0.001$; Figure 1A). Meanwhile, we showed that miR-99b-5p expression was also lower in SCLC cell line than in 16-HBE or HEK293T cell line ($P < 0.05$; Figure 1A). In addition, we analyzed

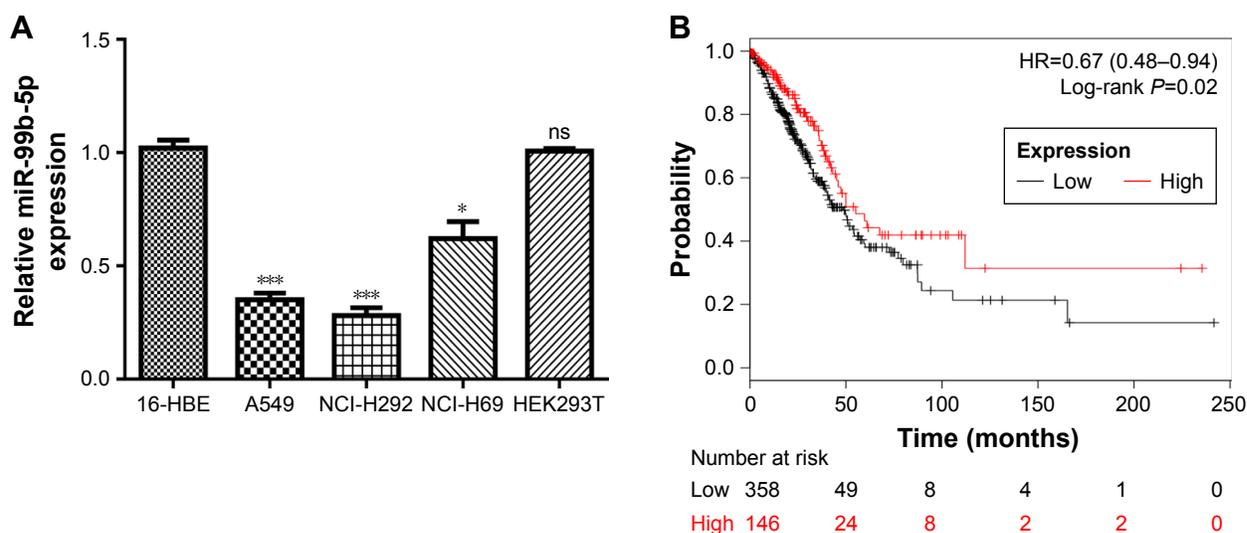


Figure 1 Expression and clinical significance of miR-99b-5p expression in NSCLC. **(A)** Expression level of miR-99b-5p in NSCLC cell lines, SCLC cell line and normal cell line. **(B)** Kaplan–Meier curve of cancer patients with high or low miR-99b-5p expression. $*P < 0.05$, $***P < 0.001$.

Abbreviations: miR-99b-5p, miRNA-99b-5p; ns, not significant; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer.

the effect of miR-99b-5p expression on overall survival of NSCLC patients and found that low miR-99b-5p expression was correlated with poor overall survival (Figure 1B). These results indicated that miR-99b-5p plays a crucial role in the carcinogenesis of NSCLC.

Overexpression of miR-99b-5p inhibits NSCLC cell proliferation, migration and invasion

We further investigated the effects of miR-99b-5p on cell proliferation, migration and invasion by introducing the synthetic miRNAs into NSCLC cell lines. We found that miR-99b-5p mimic transfection significantly enhanced the levels of miR-99b-5p in NSCLC cell lines (Figure 2A).

CCK-8 assay revealed that cellular proliferation rate of NSCLC cell lines was significantly reduced by miR-99b-5p mimic compared with NC-miRNA (Figure 2B). Similarly, wound-healing assay demonstrated that cell migration ability was inhibited by miR-99b-5p mimic (Figure 2C). We also found that NSCLC cell invasion ability was remarkably suppressed by miR-99b-5p mimic compared with NC-miRNA (Figure 2D). These results indicated that miR-99b-5p overexpression repressed NSCLC cell proliferation, migration and invasion.

FZD8 was a direct target of miR-99b-5p

TargetScan analysis results showed that miR-99b-5p could bind to the 3'-UTR of FZD8 (Figure 3A). Luciferase activity

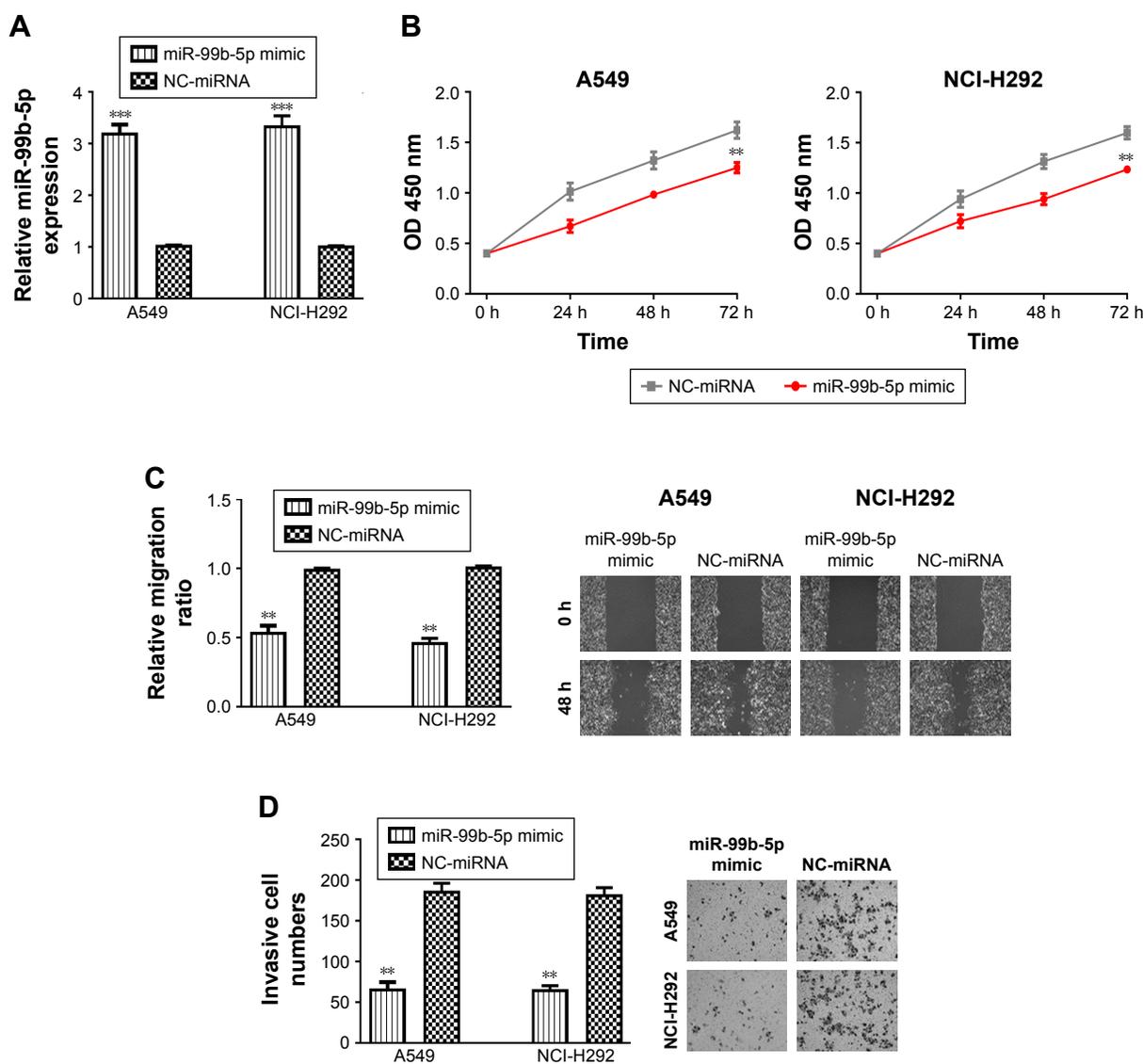


Figure 2 Overexpression of miR-99b-5p inhibits NSCLC cell proliferation, migration and invasion. (A) miR-99b-5p expression, (B) cell proliferation, (C) cell migration and (D) cell invasion in cells transfected with miR-99b-5p mimic or NC-miRNA. ** $P < 0.01$, *** $P < 0.001$.

Abbreviations: miR-99b-5p, miRNA-99b-5p; NC-miRNA, negative control miRNA; NSCLC, non-small cell lung cancer.

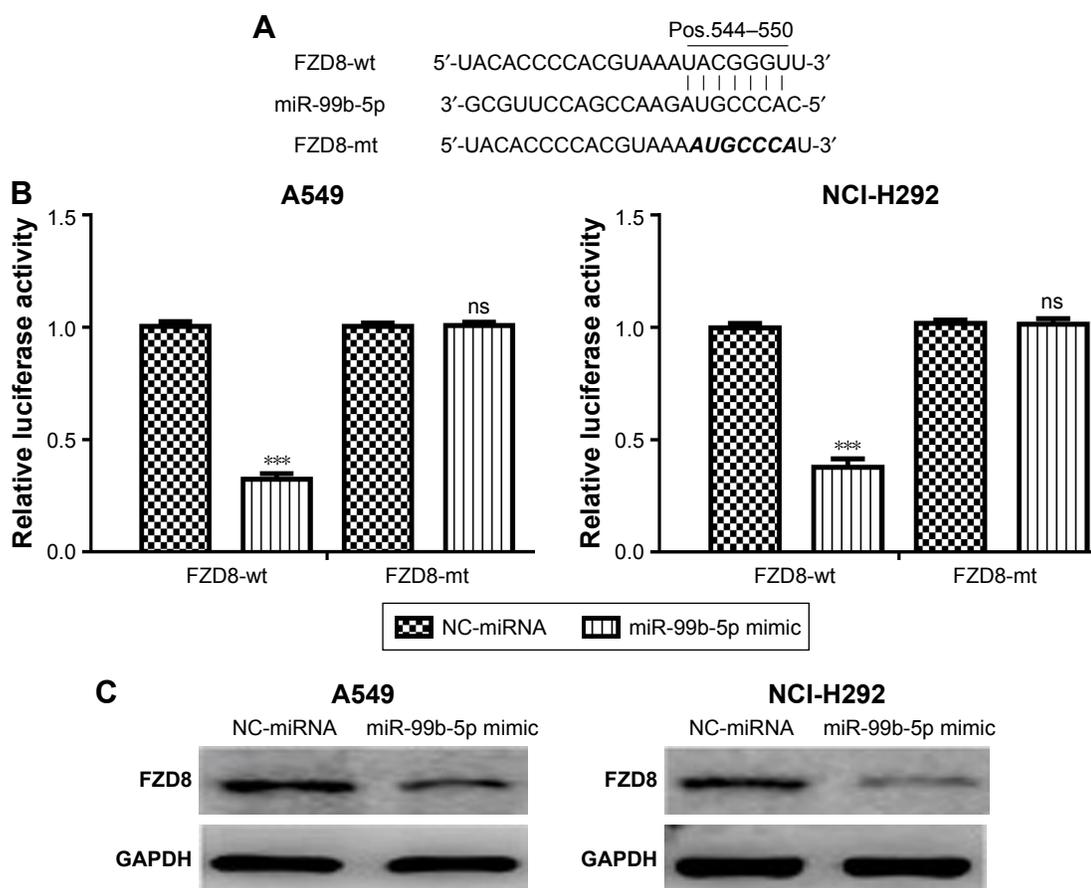


Figure 3 FZD8 was a direct target of miR-99b-5p. **(A)** The predicted miR-99b-5p binding site within the 3'-UTR of FZD8 and a mutated version are shown. **(B)** Luciferase reporter assay illustrating direct binding of miR-99b-5p to the FZD8-wt, but not FZD8-mt. **(C)** The protein expression of FZD8 was regulated by miR-99b-5p in NSCLC cells. *** $P < 0.001$.

Abbreviations: miR-99b-5p, miRNA-99b-5p; mt, mutant; NC-miRNA, negative control miRNA; ns, not significant; NSCLC, non-small cell lung cancer; wt, wild-type.

reporter assay validated that relative luciferase activity in cells transfected with FZD8-wt was repressed by miR-99b-5p mimic (Figure 3B). Furthermore, we found that FZD8 protein expression in NSCLC cells was inhibited by miR-99b-5p mimic (Figure 3C). Thus, these results implied that FZD8 was a direct target of miR-99b-5p.

FZD8 was involved in miR-99b-5p inhibitory effects on NSCLC cell behaviors

Based on the abovementioned findings, we hypothesized that miR-99b-5p regulates NSCLC cell behaviors through targeting FZD8. Western blot confirmed the overexpression status of FZD8 after pcDNA3.1-FZD8 transfection (Figure 4A). As shown in Figure 4B–D, the overexpression of FZD8 significantly increased cell proliferation, migration and invasion. Moreover, the inhibitory effects of miR-99b-5p on NSCLC cell behaviors could partially reversed by FZD8 overexpression (Figure 4B–D).

Discussion

Recent studies have investigated that biomarkers are associated with cancer progression with the aim to develop novel treatment strategies for cancer.^{17–19} Previously, miR-99b-5p was reported to display a crucial role in human cancers to regulate various cellular behaviors.^{13–15} It was found that miR-99b-5p functions as a tumor suppressor in gastric cancer through targeting insulin-like growth factor 1 receptor.¹³ Furthermore, miR-99b-5p was found to suppress colorectal cancer metastasis to liver through targeting mTOR.¹⁵ However, the role of miR-99b-5p in NSCLC remains unclear till now.

We found that miR-99b-5p expression was significantly reduced in NSCLC cell lines compared with normal cell line. Moreover, we found that low miR-99b-5p expression was a predictor of poor overall survival in NSCLC patients. These results suggested the tumor-suppressive role of miR-99b-5p in NSCLC, which is in consistent with the previous studies.^{13–15} In addition, we confirmed that the elevated expression of miR-99b-5p could inhibit NSCLC cell proliferation, migration

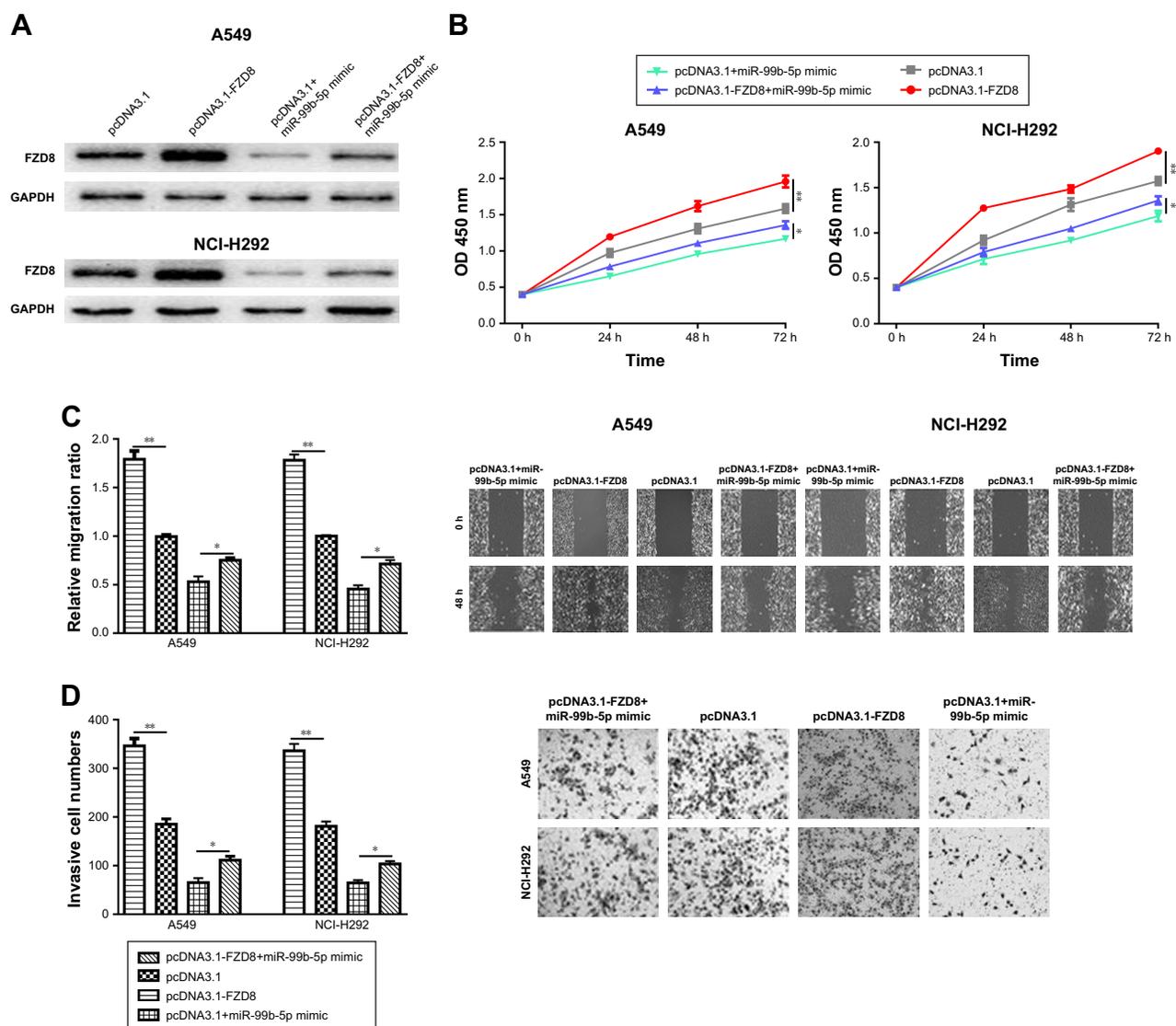


Figure 4 Overexpression of FZD8 reversed the inhibitory effects of miR-99b-5p on NSCLC cell behaviors. (A) FZD8 expression, (B) cell proliferation, (C) cell migration and (D) cell invasion in cells transfected with pcDNA3.1-FZD8, pcDNA3.1, pcDNA3.1-FZD8 and miR-99b-5p mimic or pcDNA3.1 and miR-99b-5p. * $P < 0.05$, ** $P < 0.01$. **Abbreviations:** miR-99b-5p, miRNA-99b-5p; ns, not significant; NSCLC, non-small cell lung cancer.

and invasion in vitro. These results collectively indicated that miR-99b-5p plays a crucial role in NSCLC carcinogenesis.

FZD8, a member of FZD receptor family, is reported to be a crucial receptor for the Wnt/ β -catenin signaling pathway.²⁰ FZD8 was also reported to be a target of p53 to promote prostate cancer metastasis to bone through the Wnt/ β -catenin signaling pathway.²¹ In addition, FZD8 expression was found to be directly regulated by miRNAs in human cancers.^{22,23} For example, miR-520b was found to suppress spinal osteosarcoma cell proliferation, migration and invasion via down-regulation of FZD8.²² In addition, miR-100 could suppress breast cancer cell migration and invasion by targeting FZD-8 and inhibiting Wnt/ β -catenin signaling pathway.²³

Here, by TargetScan analysis, we identified that FZD8 might be a target of miR-99b-5p. Luciferase activity reporter assay and Western blot assay validated this prediction. Moreover, rescue experiments showed that the overexpression of FZD8 could partially reversed the inhibitory effects of miR-99b-5p on NSCLC cell behaviors.

Conclusion

miR-99b-5p plays a crucial role in regulating NSCLC proliferation, migration and invasion through suppressing FZD8. These results indicated that miR-99b-5p functions as a tumor suppressor in NSCLC and might be developed as therapeutic target for NSCLC in the near future.

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Author contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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