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## miR-767-3p Inhibits Growth and Migration of Lung Adenocarcinoma Cells by Regulating CLDN18

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Claudin18 (CLDN18) is necessary for intercellular junctions and is reported to be involved in cell migration and metastasis, making it like an oncogene in various cancer types. However, the biological function and regulatory mechanisms of CLDN18 in lung adenocarcinoma are not yet clear. In this study, we found downregulation of miR-767-3p and upregulation of *CLDN18* in lung adenocarcinoma tissue and cell lines. In addition, there was a negative correlation between the expression of miR-767-3p and CLDN18 in lung adenocarcinoma. Double luciferase reporter gene analysis showed that miR-767-3p modulates the expression of CLDN18 by binding its 3'-untranslated regions (3'-UTR). Knockdown of CLDN18 results in a decrease in the growth, migration, and invasion of lung adenocarcinoma cells. Although overexpression of miR-767-3p inhibits lung adenocarcinoma cell growth and migration, these effects can be rescued by reexpressing CLDN18. In summary, the data suggest that miR-767-3p inhibits tumor cell proliferation, migration, and invasion by targeting CLDN18, providing a promising therapeutic target for lung adenocarcinoma.

**Key words:** miR-767-3p; Lung adenocarcinoma; Claudin 18 (CLDN18); Migration; Invasion

### INTRODUCTION

As one of the most common malignancies, lung cancer is the leading cause of cancer-related deaths worldwide. Lung adenocarcinoma is the most common type of lung cancer, accounting for ~35% of primary lung cancers<sup>1</sup>. Although there have been recent advances in the diagnosis of and treatment strategies for lung cancer, a significant proportion of patients with lung adenocarcinoma will eventually die; the 5-year overall survival rate is about 11%<sup>2</sup>. Therefore, it is urgent to clarify the molecular mechanism of the development of lung adenocarcinoma and identify new prognostic markers and molecular therapeutic targets to improve the diagnosis and treatment of lung adenocarcinoma.

MicroRNAs (miRNAs) are a small class of non-coding RNAs (about 20–23 nucleotides) that regulate the expression of their target genes through binding to 3'-untranslated regions (3'-UTRs) at posttranscriptional levels<sup>3</sup>. Substantial studies have shown that dysregulated miRNAs are involved in a variety of biological and pathological processes, including tumor cell proliferation, survival, apoptosis, and tumorigenesis.<sup>4,5</sup> miRNAs

commonly function as tumor suppressors or oncogenes, which play crucial roles in the development and progression of various cancers<sup>6</sup>. Recent research identified 21 miRNAs as cancer genes and 7 miRNAs as tumor suppressors in lung adenocarcinoma using high-throughput sequencing analysis<sup>7</sup>. Moreover, miR-483-5p upregulation is associated with progression of lung adenocarcinoma through promoting tumor cell epithelial–mesenchymal transition (EMT) as an oncogene<sup>8</sup>. Previously, miR-767, as an oncogene, was shown to accelerate cell proliferation and metastasis of several cancers, including bladder cancer, gastric cancer, and prostate cancer<sup>9</sup>. However, the expression and role of miR-767-3p in lung adenocarcinoma remain unclear.

In the present study, we demonstrated that miR-767-3p is downregulated in lung adenocarcinoma patients and cell lines, and negatively correlated with claudin 18 (CLDN18) expression. Upregulation of miR-767-3p inhibits tumor growth and migration by reducing the expression levels of CLDN18. These results identified a new axis consisting of miR-767-3p and CLDN18 for controlling tumor growth and migration in lung adenocarcinoma.

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## MATERIALS AND METHODS

### *Tissues and Cell Culture*

A total of 39 lung adenocarcinoma tissues and normal tissues were collected from The First Affiliated Hospital of Soochow University (Jiangsu, P.R. China). Patients were proven to be suffering from lung adenocarcinoma and received chemotherapy or radiotherapy. Cell lines of lung adenocarcinoma (H1975, HCC827, H1650, and H441), and SV40-immortalized nontumorigenic human bronchial epithelial cells (BEAS-2B) used as a control, were obtained from the Chinese Academy of Sciences Cell Bank of Type Culture Collection (CBTCCAS, Shanghai, P.R. China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) or Roswell Park Memorial Institute (RPMI)-1640 medium, which was supplemented with 10% fetal bovine serum (FBS) and 100 mg/ml streptomycin/penicillin. Cells were maintained in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

### *RNA Isolation and Qualitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)*

Total RNA was extracted from lung adenocarcinoma specimens and cell lines by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). PCR was performed using the SYBR Green PCR Kit (Invitrogen) from Applied Biosystems (Foster City, CA, USA). The following were the primers used: *CLDN18*, 5'-ACATGCTGGTGAAGTAACTTCTG-3' (forward) and 5'-AAATGTGTACCTGGTCTGAACAG-3' (reverse); glyceraldehydes 3-phosphate dehydrogenase (*GAPDH*), 5'-CTCACC GGATGCACCAATGTT-3' (forward) and 5'-CGCGTTGCTCACAATGTTTCAT-3' (reverse). TaqMan MicroRNA Assay was used to reverse the translation of RNA. YM500v2 meta-analysis (<http://ngs.yu.edu.tw/ym500v2/index.php>) was performed to determine miRNA expression in lung adenocarcinoma specimens and normal tissue. After identifying miR-767-3p as an miRNA whose expression was significantly altered in lung adenocarcinoma, TargetScan (<http://www.targetscan.org>) was used to investigate potential binding partners.

### *Western Blot and Immunohistochemistry Staining Assay*

Target protein expression was assessed by Western blotting analysis of total cell lysates in radioimmunoprecipitation assay (RIPA) buffer in the presence of rabbit antibodies to CLDN18 and  $\beta$ -actin (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The signal was analyzed using the chemiluminescence detection system (Millipore, Braunschweig, Germany) and visualized with the ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA). In immunohistochemistry (IHC) assays, the samples were cut into 4-mm slices. The tissue samples were

incubated with CLDN18 antibody and then photographed with an Olympus microscope (Tokyo, Japan).

### *Luciferase Assay*

Mutant (MUT) and wild-type (WT) sequences of *CLDN18* 3'-UTR were amplified by PCR and cloned into control vector. 293T, H195, and HCC827 cells were cotransfected with WT or MUT of *CLDN18* 3'-UTR, miR-767-3p, or a negative control miR (miR-NC). Cells were obtained 48 h after transfection. The luciferase activity was performed using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

### *Cell Proliferation Assays*

Cell viability was analyzed by CellTiter 96<sup>®</sup> Aqueous One Solution Cell Proliferation Assay (Promega). Briefly after transfection, cells were seeded in 96-well cell culture plates and cultured for 24, 48, 72, and 96 h. Then 20  $\mu$ l of One Solution reagent was added to the 96-well cell culture plates and incubated for 4 h. The absorbance was measured at 490 nm using a Synergy<sup>™</sup> HT multimode microplate reader (Bio-Tek, Winooski, VT, USA)<sup>10</sup>.

### *Wound Healing Migration Assay*

Cells ( $1 \times 10^5$  H1975 or HCC827) were seeded into six-well plates. The confluent monolayers of cells were scratched with a 200- $\mu$ l pipette tip, and the wound closures of cells were observed by measuring the size of the initial wound and comparing the size of the wound after 24 h<sup>11</sup>.

### *Transwell Invasion Assay*

Cell invasion was performed using Transwell chambers. Cells were seeded in the upper Transwell chamber of 24-well plates containing serum-free medium. The lower chamber contained medium with 10% serum for 24 h. The invasion cells at the bottom of the chamber were stained and counted<sup>12</sup>.

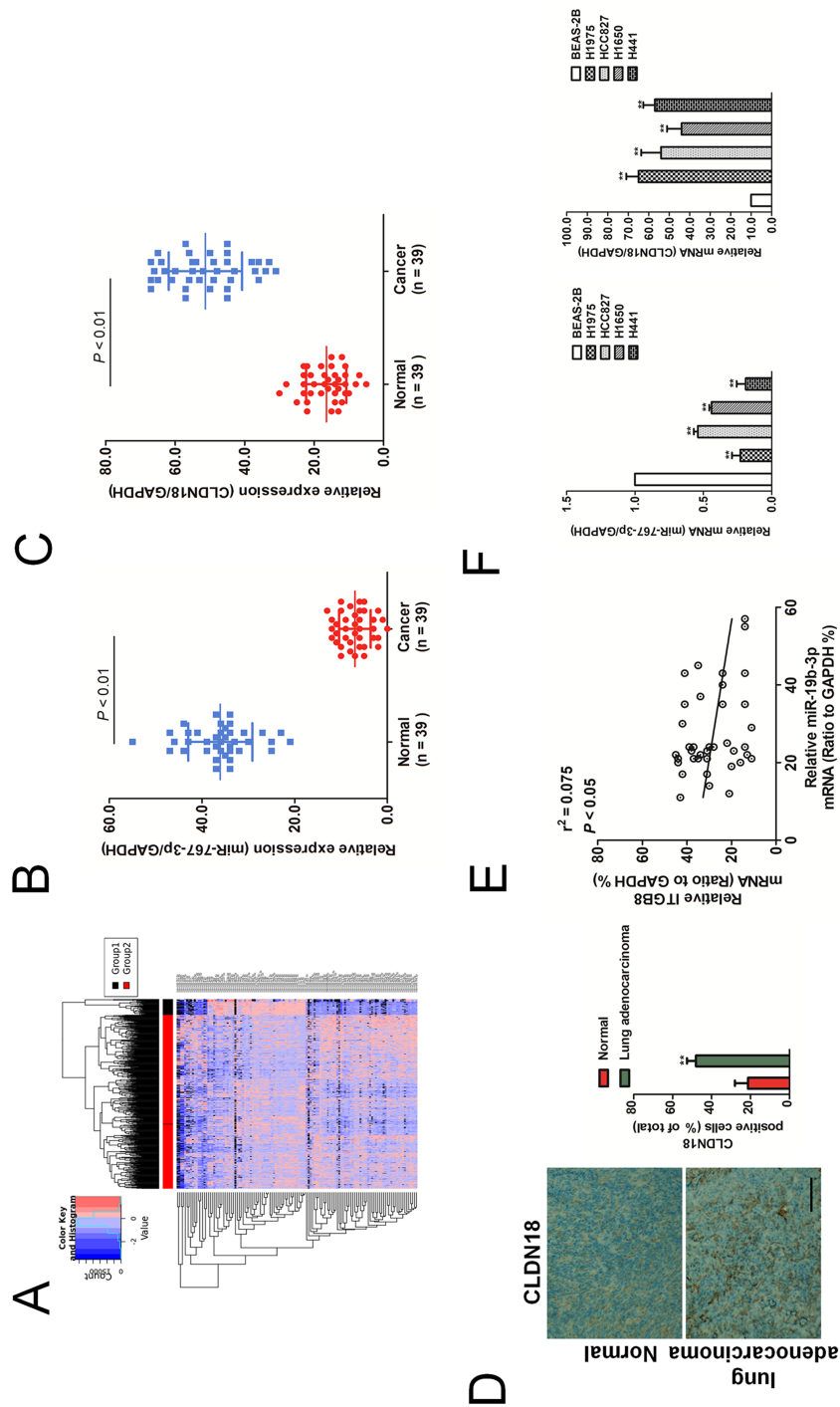
### *In Vivo Assay*

H1975 cells were transfected with control short hairpin RNA (shRNA; shControl) or shCLDN18. Two-week-old mice (SLAC Laboratory Animal Co. Ltd., Shanghai, P.R. China) were subcutaneously inoculated with 0.1 ml of a  $1 \times 10^6$  H1975 cell suspension (six mice per group). Every 3 days, measurements were made to assess tumor volume and calculate tumor volume by the formula  $(\text{length} \times \text{width}^2)/2$ <sup>13</sup>. Mice were sacrificed after 25 days.

### *Statistical Analysis*

A Student's *t*-test and one-way ANOVA was performed to analyze the two different groups. Data represent means  $\pm$  SD of three separate experiments. Statistical





**Figure 1.** MicroRNA 767-3p (miR-767-3p) was downregulated in lung adenocarcinoma and is negatively correlated with claudin 18 (CLDN18) expression. (A) miRNA meta-analysis in YIM500v2 (<http://ngs.yim.edu.tw/yim500v2/index.php>) was performed to detect the differential expression of miRNAs in lung adenocarcinoma compared to the control normal tissue. Group 1: normal tissue, group 2: lung adenocarcinoma. (B) The levels of miR-767-3p in 39 cases of lung adenocarcinoma and 20 cases of normal tissues were detected by qualitative reverse transcriptase polymerase chain reaction (qRT-PCR). (C) qRT-PCR assay was performed to detect the levels of *CLDN18* in lung adenocarcinoma tissues and normal tissues. (D) Immunohistochemistry (IHC) staining was used to measure the expression of CLDN18 in lung adenocarcinoma and normal tissues. Scale bar: 200  $\mu$ M. Data are expressed as mean  $\pm$  SD.  $^{**}p < 0.01$ , compared with normal tissues. (E) The level of miR-767-3p was negatively correlated with *CLDN18* in lung adenocarcinoma. (F) The RNA levels of miR-767-3p and CLDN18 were measured in a panel of lung adenocarcinoma cell lines. Data are expressed as mean  $\pm$  SD.  $^{**}p < 0.01$ , compared to BEAS-2B cells.

analyses were performed by the GraphPad Prism 5 software. A value of  $p < 0.05$  was considered statistically significant.

## RESULTS

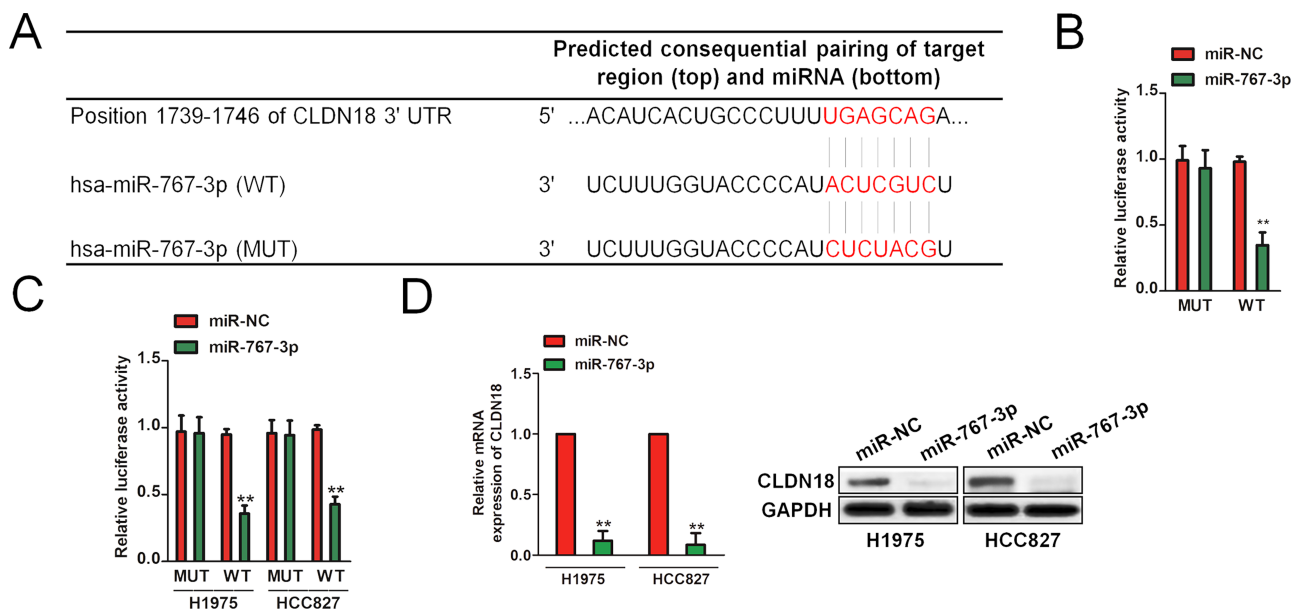
### *miR-767-3p Was Downregulated in Lung Adenocarcinoma*

YM500v2 meta-analysis was performed to identify miRNAs that were differentially expressed in lung adenocarcinoma tissue and normal solid tissue (Fig. 1A). A total of 2,578 miRNAs were significantly altered in lung adenocarcinoma (1,697 were upregulated and 881 were downregulated) compared to normal tissue. Of the 1,697 upregulated miRNAs, 613 miRNAs were identified as upregulated to infinitely increased levels compared to the very low expression levels in the normal group of these miRNAs. In the 881 downregulated miRNAs, we found that miR-767-3p ranked first among them. In order to determine the levels of both miR-767-3p and *CLDN18* in lung adenocarcinoma, we collected 39 cases of lung adenocarcinoma and 20 normal tissues. As shown in Figure 1B, miR-767-3p levels were significantly downregulated in the lung adenocarcinoma tissue compared with the normal tissue ( $p < 0.05$ ). However, *CLDN18* expression was also higher in lung adenocarcinoma than in normal tissues ( $p < 0.05$ ) (Fig. 1C). IHC staining

was performed to determine the expression of *CLDN18* (Fig. 1D). Interestingly, a strong negative relationship between miR-767-3p and *CLDN18* was observed in lung adenocarcinoma (Fig. 1E). In addition, a group of lung adenocarcinoma cell lines, including H1975 and HCC827, was obtained to explore the expression pattern of miR-767-3p and *CLDN18*, and similar negative correlations were found in the cell lines (Fig. 1F).

### *CLDN18 Was a Target Gene of miR-767-3p*

In order to further explore the relationship between *CLDN18* and miR-767-3p, we consulted TargetScan (<http://www.targetscan.org>) to confirm that *CLDN18* is one of the miR-767-3p target genes (Fig. 2A). To reveal the correlation between miR-767-3p and *CLDN18*, we cotransfected 293T cells with *CLDN18* WT-, *CLDN18* MUT-, miR-767-3p-, or miR-NC-containing vectors. As shown in Figure 2B, the luciferase reporter assays showed that overexpression of miR-767-3p reduced the activity of the *CLDN18* WT, whereas the effect of miR-767-3p was eliminated in the cells transfected with *CLDN18* MUT. Similar results were obtained in H1975 and HCC827 cells (Fig. 2C). Finally, qRT-PCR and Western blot analysis were performed to further confirm the expression level of *CLDN18* in miR-767-3p-overexpressing cells and demonstrate that overexpression of miR-767-3p inhibited the expression of *CLDN18* (Fig. 2D). In summary, these



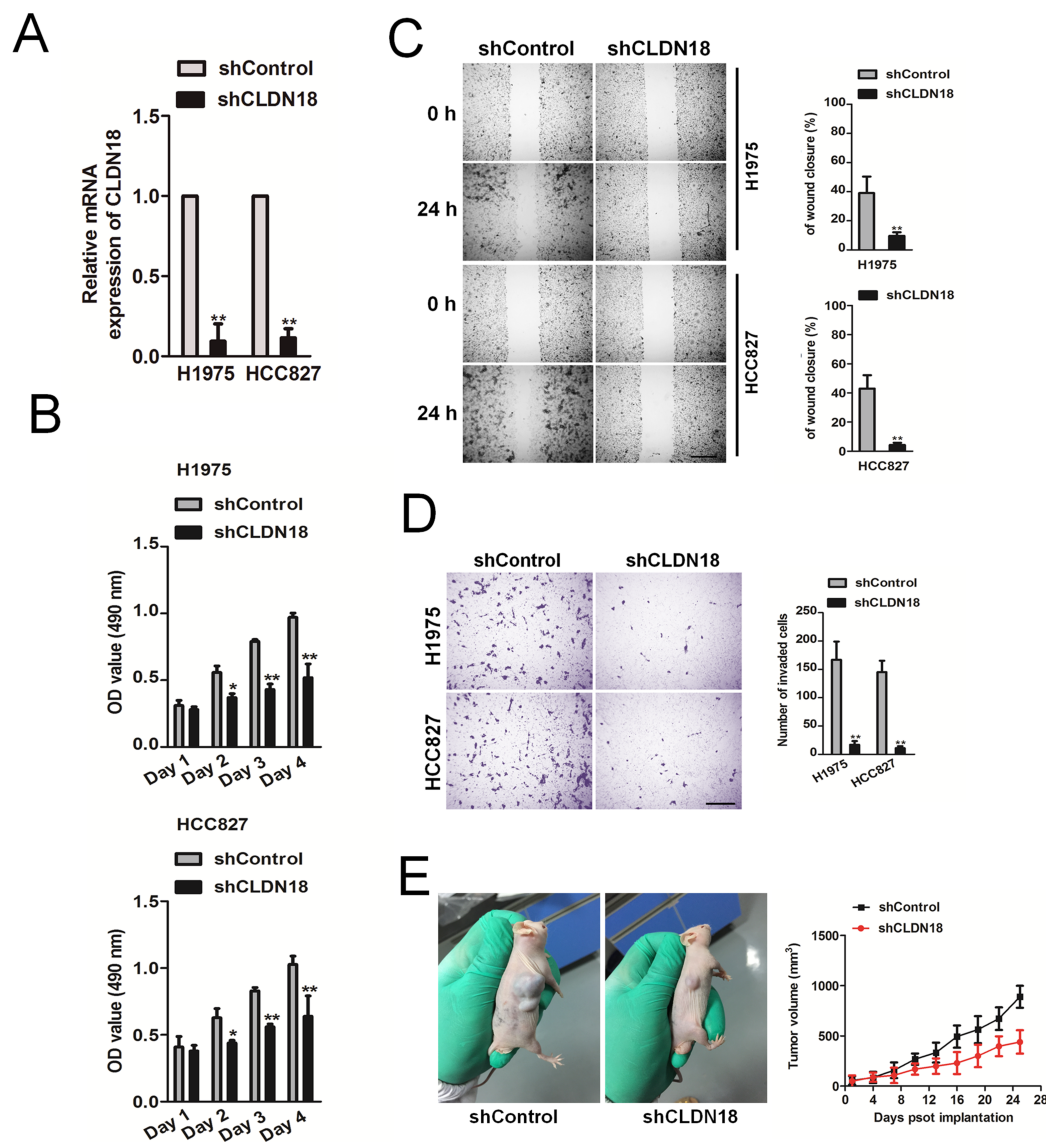
**Figure 2.** *CLDN18* is the target gene of miR-767-3p. (A) The sequence of the miR-767-3p binding site in the 3'-untranslated region (3'-UTR) of *CLDN18*. (B) Wild-type (WT) and mutant (MUT) sequences of *CLDN18* 3'-UTR, miR-767-3p, and miR-negative control (NC) were cotransfected into 293T cells. The luciferase activity was measured 48 h after transfection. Data are expressed as mean  $\pm$  SD. \*\* $p < 0.01$ , compared with miR-NC. (C) Dual-luciferase reporter assay was performed in both H1975 and HCC827 cells. Data are expressed as mean  $\pm$  SD. \*\* $p < 0.01$ , compared with miR-NC. (D) Cells were transfected with miR-767-3p and its corresponding control. The expression of *CLDN18* was detected by qRT-PCR (left) and Western blot assays (right). Data are expressed as mean  $\pm$  SD. \*\* $p < 0.01$ , compared with miR-NC.

results indicated that *CLDN18* was a potential target of miR-767-3p in lung adenocarcinoma.

*Downregulation of CLDN18 Inhibits Tumor Growth and Migration in Lung Adenocarcinoma*

To further investigate the role of *CLDN18* in lung adenocarcinoma, lung adenocarcinoma cell lines H195

and HCC827 were transfected with shRNA to knock down *CLDN18* expression. As expected, the level of *CLDN18* was significantly suppressed in *CLDN18*-specific shRNA (sh*CLDN18*)-transfected H195 and HCC827 cells (Fig. 3A). Then cell proliferation assays were performed to evaluate the effect of *CLDN18* on cell growth in vitro, which demonstrated that knockdown

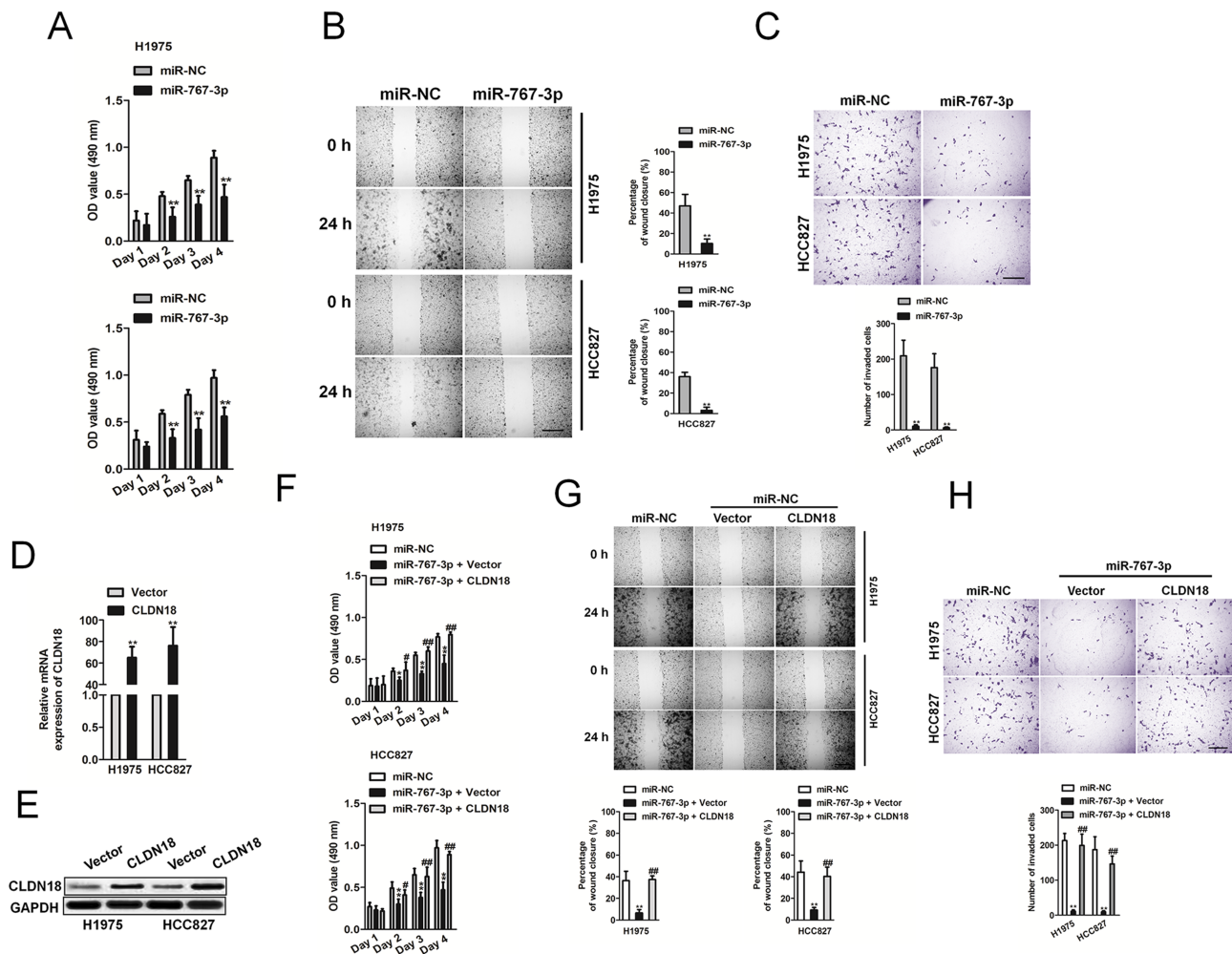


**Figure 3.** Downregulation of *CLDN18* inhibits tumor growth, migration, and invasion in lung adenocarcinoma cells. (A) *CLDN18* mRNA levels were determined in cells transfected with short hairpin RNA (shRNA) control (shControl) or *CLDN18*-specific shRNA (sh*CLDN18*) by qRT-PCR. Data are expressed as mean  $\pm$  SD. \*\* $p < 0.01$ , compared with shControl. (B). The effect of *CLDN18* knock-down on cell proliferation was assessed in H1975 and HCC827 cells. Data are expressed as mean  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , compared with shControl. (C) After sh*CLDN18* transfection, cell migration was measured by wound healing assay. Data are expressed as mean  $\pm$  SD. \*\* $p < 0.01$ , compared with shControl. Scale bar: 200  $\mu$ m. (D). After sh*CLDN18* transfection, cell invasion was measured by Transwell invasion assay. Data are expressed as mean  $\pm$  SD. \*\* $p < 0.01$ , compared with shControl. Scale bar: 200  $\mu$ m. (E). Mice xenograft tumor model was established by subcutaneous inoculation with sh*CLDN18* H1975 cells or shControl cells (six mice per group).

of *CLDN18* inhibited the proliferation of H195 and HCC827 cells (Fig. 3B). Wound healing assay and Transwell analysis revealed that silencing *CLDN18* inhibited migration (Fig. 3C) and invasion (Fig. 3D) of H195 and HCC827 cells. In addition, we established a nude mouse xenograft model to assess the effect of *CLDN18* on the growth of lung adenocarcinoma cells H1975 *in vivo*. As expected, the tumor volume of the *CLDN18*-silencing group was significantly reduced, compared to the control group (Fig. 3E).

### *miR-767-3p* Suppressed Growth and Migration of Lung Adenocarcinoma Cells by Targeting *CLDN18*

Based on the results above, we hypothesized that *miR-767-3p* downregulation could inhibit tumor growth and migration by modulating *CLDN18*. To further validate this idea, we transfected H195 and HCC827 cells with *miR-NC* or *miR-767-3p*. The results showed that the *miR-767-3p* inhibited the proliferation of H195 and HCC827 after transfection (Fig. 4A). Wound healing and



**Figure 4.** *miR-767-3p* inhibits lung adenocarcinoma cell growth and migration by targeting *CLDN18*. (A). H195 and HCC827 cells were transfected with *miR-767-3p*, and cell proliferation was analyzed. Data are expressed as mean  $\pm$  SD. \*\* $p < 0.01$ , compared with *miR-NC*. (B) Cells were transfected with *miR-767-3p* or *miR-NC* to determine the ability to migrate. Data are expressed as mean  $\pm$  SD. \*\* $p < 0.01$ , compared with *miR-NC*. (C) Cells were transfected with *miR-767-3p* or *miR-NC* to determine the invasion ability *in vitro*. Data are expressed as mean  $\pm$  SD. \*\* $p < 0.01$ , compared with *miR-NC*. (D). H195 and HCC827 were transfected with a vector containing *CLDN18*, and the mRNA level of *CLDN18* was assessed by qRT-PCR. Data are expressed as mean  $\pm$  SD. \*\* $p < 0.01$ , compared with vector. (E). H195 and HCC827 cells were transfected with a vector containing *CLDN18*, and the expression of *CLDN18* was analyzed by Western blotting analysis. (F). H195 and HCC827 cells were transfected with *miR-767-3p* or *CLDN18*, and cell proliferation was analyzed. (G). H195 and HCC827 were cotransfected with *miR-767-3p* or *CLDN18*, and cell migration was analyzed in wound healing assay. (H) Transwell assay was performed to assess the effect of *miR-767-3p* and *CLDN18* on cell invasion. Data are expressed as mean  $\pm$  SD. \* $p < 0.05$  and \*\* $p < 0.01$ , compared with *miR-NC*. # $p < 0.05$  and ### $p < 0.01$ , compared with *miR-767-3p* + vector.



Transwell assays suggested that the migration ability (Fig. 4B) and invasion capacity (Fig. 4C) of miR-767-3p-overexpressing cells were significantly inhibited. To determine the effect of CLDN18 on miR-767-3p-regulated lung adenocarcinoma cell growth and migration, *CLDN18* was stably transfected into H195 and HCC827 cells, and the mRNA and protein levels of CLDN18 were analyzed by qRT-PCR (Fig. 4D) and Western blotting (Fig. 4E). In fact, ectopic expression of *CLDN18* rescued cell proliferation, migration, and invasion, which had been inhibited by miR-767-3p (Fig. 4F–H).

## DISCUSSION

It has been shown that miRNAs function as regulatory factors for specific gene expression and thus affect tumorigenesis, migration, and metastasis<sup>14</sup>. Previous studies have revealed that miR-767-3p acts as an inhibitor in various types of cancers<sup>15</sup>. In this study, we found that miR-767-3p was downregulated in lung adenocarcinoma tissue and a panel of cell lines, suggesting that miR-767-3p may inhibit the development of lung adenocarcinoma.

CLDN18, which is encoded by the *CLDN18* gene, is a transmembrane protein that plays an important role in tight junctions<sup>16</sup>. Substantive research has demonstrated that abnormal expression of CLDN18 is associated with tumor progression<sup>17</sup>. Herein we revealed that CLDN18 was upregulated in lung adenocarcinoma tissue compared to normal tissue, and similar results were found in a panel of lung adenocarcinoma cell lines. A previous study revealed that loss of CLDN18 suppresses tumor cell migration and invasion, which will be the initial step in tumor metastasis<sup>18</sup>. As an oncogene, CLDN18 is an early stage marker of pancreatic carcinogenesis. However, the role of CLDN18 in tumor growth, migration, and the regulatory mechanism in lung adenocarcinoma needs further investigation. Interestingly, there was a negative correlation between CLDN18 and miR-767-3p expression in lung adenocarcinoma. In fact, the results of luciferase assays showed that miR-767-3p downregulated the expression of CLDN18 by direct binding to CLDN18 3'-UTR, indicating that CLDN18 was the target of miR-767-3p.

We then explored the biological effects of CLDN18 in lung adenocarcinoma cells by transfection with specific shRNA targeting CLDN18. In vitro and in vivo studies have shown that CLDN18 knockdown inhibits lung adenocarcinoma cell growth, migration, and invasion. A previous study suggested that CLDN18 is specifically abnormally expressed in stomach and lung cancers<sup>19</sup>. Therefore, CLDN18 may be a potential therapeutic target for lung adenocarcinoma. In addition, functional assays showed that miR-767-3p overexpression inhibited tumor growth, migration, and invasion. Notably, overexpression

of CLDN18 reversed miR-767-3p-mediated inhibitory effects, suggesting that miR-767-3p regulates tumor growth, migration, and invasion by targeting CLDN18 in lung adenocarcinoma.

In conclusion, our data suggest a significant correlation between miR-767-3p and CLDN18 in lung adenocarcinoma. Downregulation of miR-767-3p and overexpression of CLDN18 were observed in lung adenocarcinoma and cell lines. Overexpression of miR-767-3p inhibits cell proliferation, migration, and invasion of lung adenocarcinoma cells by targeting CLDN18. Therefore, we highlighted the interaction of miR-767-3p and CLDN18 in lung adenocarcinoma development, providing new targets for exploring new therapeutic strategies.

**ACKNOWLEDGMENT:** *The authors declare no conflicts of interest.*

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