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miR-342-3p Regulates the Proliferation and Apoptosis of NSCLC Cells by Targeting *BCL-2*

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Abstract

microRNA-342-3p plays an important role in tumor occurrence and development. However, the expression pattern and roles of microRNA-342-3p in nonsmall cell lung cancer remain poorly understood. In the current study, we explored the roles and underlying mechanisms of microRNA-342-3p in nonsmall cell lung cancer via gain- and loss-of-function analyses. We used quantitative reverse-transcription-polymerase chain reaction and western blotting assays to measure the expression levels of microRNA-342-3p in non-small-cell lung cancer and B-cell lymphoma-2. Furthermore, we used small interfering RNA and RNA mimics to analyze the functions and underlying mechanisms of microRNA-342-3p in nonsmall cell lung cancer cells. A luciferase reporter assay was performed to evaluate the direct binding site of the 5′-untranslated region of B-cell lymphoma-2 targeted by microRNA-342-3p. We found that the expression of microRNA-342-3p was significantly lower in nonsmall cell lung cancer cells and tissues than in normal cells and tissues. The upregulation of microRNA-342-3p suppressed cell proliferation while promoting apoptosis in H1975, H460, and H226 cells. The overexpression of microRNA-342-3p in nonsmall cell lung cancer cells led to the downregulation of mRNA and protein levels in B-cell lymphoma-2 cells. Thus, B-cell lymphoma-2 was identified as a direct target of microRNA-342-3p. These findings indicate that microRNA-342-3p inhibits the growth of nonsmall cell lung cancer by repressing the expression of B-cell lymphoma-2, which suggests that microRNA-342-3p could be a potential target for the treatment of nonsmall cell lung cancer.

Keywords

nonsmall cell lung cancer, microRNA, overexpression, cell proliferation, apoptosis

Abbreviations

AGR2, anterior gradient 2; BCL-2, B-cell lymphoma-2; FOXQ1, forkhead box Q1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hsa-miRNA-342-3p, homo sapiens-microRNA-342-3p; hUC-MSCs, human umbilical cord-derived mesenchymal stem cells; LASP1, LIM and SH3 protein 1; miRNA, microRNA; miR-342-3p, microRNA-342-3p; miR-NC, microRNA-negative control; MUT, mutant BCL-2; NSCLC, nonsmall cell lung cancer; OSCC, oral squamous cell carcinoma; PBS, phosphate-buffered saline; PI, propidium iodide; PVDF, polyvinylidene fluoride; qRT-PCR, quantitative reverse-transcription-polymerase chain reaction—PCR; RIPA, radioimmunoprecipitation assay; RPMI, Roswell Park Memorial Institute; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; siRNA, small interfering RNA; TBST, Tris-buffered saline + Tween 20; UTR, untranslated region; WT, wild-type BCL-2.

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Introduction

Lung cancer is one of the most destructive cancers worldwide and is characterized by its aggressiveness, high incidence, and lack of effective treatments.[1] Nonsmall cell lung cancer (NSCLC) is the main subtype of lung cancer, accounting for up to 85% of all lung cancers, with a 5-year survival rate of approximately 15%.[2] Although remarkable progress has been made in the treatment of NSCLC in recent decades, the prognosis remains poor due to the high rates of metastasis, recurrence,

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and drug resistance in patients.[3] Thus, there is a great demand to elucidate the underlying molecular mechanisms of cell growth and metastasis in NSCLC to develop new therapies and identify novel therapeutic targets for NSCLC treatment.

MicroRNAs (miRNAs) are endogenous, noncoding, single-stranded RNAs, approximately 20 to 24 nucleotides in length, that regulate gene expression at the posttranscriptional level by binding to the 3′-untranslated region (3′-UTR) of target mRNAs.[4] Dysregulation of miRNA is involved in several biological processes, including development, angiogenesis, cell differentiation, cell proliferation, metastasis, and apoptosis.[5] MiRNAs often exhibit abnormal expression in human malignant tumors and function as either tumor suppressor genes or oncogenes according to the biological role of their target genes.[6] MiRNAs play significant roles in the occurrence and progression of certain types of cancers, such as colorectal cancer, breast cancer, and lung cancer.[7–9] Dozens of miRNAs, including miR-22, miR-143/145, and miR-34, play essential roles in lung tumorigenesis by regulating key oncogenes or tumor suppressor genes.[10,11] Therefore, it is of great significance to explore the functions and potential applications of miRNAs in tumor biology.

MiRNA-342-3p correlates with the hedgehog signaling pathway of human umbilical cord-derived mesenchymal stem cells (hUC-MSCs)[12] and potentiates gemcitabine resistance in pancreatic ductal adenocarcinoma.[13] However, the biological functional role of miR-342-3p and its involvement in NSCLC progression have not been evaluated. In this study, we focused on miR-342-3p and its signaling pathway, and found that *BCL-2* is a direct functional target of miR-342-3p in NSCLC cells (H1975, H460, and H226). miR-342-3p was also found to suppress NSCLC cell growth by inhibiting *BCL-2* expression. Such findings will improve the current understanding of the pathogenesis of NSCLC and aid in the identification of new therapeutic targets for the treatment of NSCLC.

Materials and Methods

Human NSCLC Tissue Samples

A total of 40 patients with NSCLC were recruited from our hospital. Tissue samples were collected from the cancerous tissues and adjacent normal tissues during surgery, immediately frozen in liquid nitrogen, and stored at −80°C until analysis. None of the patients had received radiotherapy or chemotherapy before surgery. This study was approved by our hospital ethics committee.

Cell Lines and Cell Culture

Three human NSCLC cell lines (H1975, H460, and H226) and one normal human lung epithelial cell line (BEAS-2B) were purchased from the American Type Culture Collection. All cells were cultured in an incubator with 5% CO_2 at 37°C in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum.

RNA Extraction and qRT-PCR

Total RNA was extracted from tissue samples or cell lines using the TRIzol Total RNA Isolation Reagent (Sigma-Aldrich, Saint Louis) according to the manufacturer's instructions. cDNA was synthesized from the extracted RNA using gene-specific primers and the SuperScript III Reverse Transcriptase Kit (Sigma-Aldrich, Saint Louis), according to the manufacturer's instructions.

Quantitative reverse-transcription-polymerase chain reaction (qRT-PCR) was performed using SYBR Premix Ex Taq with specific primers on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. The sequences of the primers used herein are as follows: miRNA-342-3p forward: 5′-GTGCTATCTGTGATTGAGGGA-3′, miRNA-342-3p reverse: 5′-CGGGTGCGATTTCTGTG-3′; *BCL-2* forward: 5′-TGGGGTCATGTGTGTGG-3′, *BCL-2* reverse: 5′-GGTTC AGGTACTCAGTCATCC-3′; GAPDH forward: 5′-GACAGT CAGCCGCATCTTCT-3′, GAPDH reverse: 5′-TTAAAAGC AGCCCTGGTGAC-3′. The following qPCR cycling conditions were employed: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 56°C for 30 s. Data analysis was performed using the $2^{-\Delta\Delta}$Ct method.

Western Blotting Analysis

Cells were washed in phosphate-buffered saline (PBS) and lysed in radioimmunoprecipitation assay (RIPA) lysis buffer for 30 min on ice. Ten percentage sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate 35 μg of protein, which was transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore). After blocking in bovine serum albumin (0.1% Tween 20 + 5% w/v in PBS) for 1 h at room temperature, the proteins were probed with primary antibodies, including anti-Bcl-2 (ab32124) and anti-GAPDH (ab9485, Abcam), at the recommended dilution (1:1000) at 4°C overnight. After washing in Tris-buffered saline + Tween 20 (TBST), the membranes were further incubated with horseradish peroxidase-conjugated secondary antibodies, including goat anti-rabbit IgG (ab205718), at the recommended dilution (1:3000) for 1 h at room temperature. Protein was detected using the Image Reader LAS-4000 (Fujifilm).

MiRNA and siRNA Transfection

Endogenous miRNA-342-3p mimics, miRNA-342-3p inhibitors, scrambled microRNA-negative control (miR-NC), and small interfering RNA (siRNA) were purchased from GenePharma. Cells (3×10^5) were seeded into 6-well plates. At 12 h after plating, the cells were transfected with 50 nM miRNA-342-3p mimics, miRNA-342-3p inhibitor, or miR-NC using Lipofectamine 2000 according to the manufacturer's protocol. *BCL-2* expression was knocked down using si-BCL-2. si-BCL-2 (5′-UGUGGA

UGACUGAGUACCUGA-3′) or control-siRNA (5′-ATCCAC TACCGTTGTTATAGGTG-3′) was diluted to 100 nM with Opti-MEM medium(Invitrogen, Carlsbad, CA, USA), transfected using Lipofectamine 2000, and incubated for 30 min followed by incubation with the cells for 36 h. The transfected cells were used for subsequent experiments.

Cell Proliferation Assays

For the cell proliferation assays, the transfected cells were seeded at a density of 5000 cells/well in 96-well culture plates. Thereafter, cell proliferation was determined using the CCK-8 assay kit, according to the manufacturer's instructions. The CCK-8 solution was added (10 µL/well) to each well and incubated at 37°C for 1.5 h. Absorbance in each well was measured immediately at 450 nm using a microplate reader.

Cell Apoptosis Assays

For the apoptosis assays, the transfected cells were detected using flow cytometry with double staining using an Annexin V/propidium iodide (PI) kit (BD Biosciences). The washed cells (2×10^5) were resuspended in 100 µL binding buffer; 5 µL Annexin V and 5 µL PI were also added. After incubation in the dark for 15 min, the stained cells were analyzed using a Cytomics FC 500 flow cytometer (Beckman Coulter), and the data were analyzed using Cell Quest software. The %age of apoptotic cells was calculated and compared between the groups.

Target Prediction and Luciferase Reporter Assays

Bioinformatics analysis was performed using web-based miRNA programs, such as TargetScan (http://www.targetscan.org/), miRanda (http://www.microrna.org), and miRDB (http://www.mirdb.org/). As a result, it was predicted that homo sapiens-microRNA-342-3p (hsa-miRNA-342-3p) should bind to the 3′-UTR of *BCL-2*. The wild-type *BCL-2* (3'-UTR-WT) and mutant-type *BCL-2* (3'-UTR-MUT) were independently cloned into a pGL3 luciferase reporter vector (Promega Corporation) containing the Renilla luciferase gene. For the luciferase assay, HEK 293T cells (Cell Bank of Type Culture Collection of Chinese Academy of Sciences) were co-transfected with the wild-type luciferase reporter plasmid and miRNA-342-3p mimics or miR-NC mimic using Lipofectamine 2000. Firefly and Renilla luciferase activities were measured 48 h post-transfection using a Dual-Luciferase Reporter Assay System (Promega Corporation), according to the manufacturer's protocol. Firefly luciferase activity was normalized to Renilla luciferase activity in each well to control the transfection efficiency.

Tumorigenicity and Metastasis Assay In Vivo

All procedures of this experiment were performed in accordance with the ARRIVE guidelines and the Guide for the Care and Use of Laboratory Animals. The miRNA-342-3p inhibitor or miRNA-342-3p-NC stable cells (1.5×10^6) were injected subcutaneously into the right flank of 3- to 5-week-old BALB/c nude mice. Tumor size was recorded by measuring the length (L) and width (W) with calipers every 7 days after transfection, and the volumes (V) were calculated using the formula: $V = (L \times W^2)/2$. Mice were sacrificed by cervical dislocation on day 42, and the tumors were excised and snap-frozen for RNA and protein extraction.

Statistical Analysis

All data are expressed as mean ± SD. Statistical analysis was performed using the Student's t-test to determine the significance. A 2-sided P value ($P < .05$) was considered to indicate a statistically significant difference. *, **, and *** indicated statistical significance when the P values were .05, .01, and .001, respectively. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software) and SPSS (version 16.0; IBM).

Results

miRNA-342-3p is Downregulated in NSCLC Tissues and Cell Lines

Using qRT-PCR, the expression levels of miRNA-342-3p were detected in 40 pairs of human NSCLC tumor tissues and matched adjacent normal tissues. Based on the results, the expression levels of miRNA-342-3p in tumor tissues were significantly lower than those in the noncancerous controls (Figure 1A). Consistent with this result, all NSCLC cell lines (H1975, H460, and H226) had significantly lower miRNA-342-3p levels than the normal human bronchial epithelial cell line (BEAS-2B) (Figure 1B). Such findings suggest that miRNA-342 -3p is significantly downregulated in human NSCLC tissues and cell lines. To investigate the clinical significance of miRNA-342-3p downregulation in NSCLC, we further analyzed the relationship between clinicopathologic features and miRNA-342-3p expression levels in NSCLC cases. Importantly, we found that downregulation of miRNA-342-3p expression was associated with larger tumor size ($P < .01$, Table 1).

miRNA-342-3p Inhibits the Proliferation of NSCLC Cells and Induces Apoptosis

We further investigated the biological function of miRNA-342-3p in NSCLC cells. In H1975, H460, and H226 cells, the exogenous expression level of miRNA-342-3p was significantly increased after transfection with the miRNA-342-3p mimic compared to the miR-NC (Figure 2A). Transfection efficiency was determined using qRT-PCR. Based on the CCK-8 assays, the increased expression of miRNA-342-3p significantly inhibited the proliferative abilities of H1975, H460, and H226 cells (Figure 2B to D). A flow cytometry assay was performed to determine the effects of miRNA-342-3p on the apoptosis of NSCLC cells. The results revealed that the apoptosis %age of miRNA-342-3p cells (15%) was 3-fold greater than that of

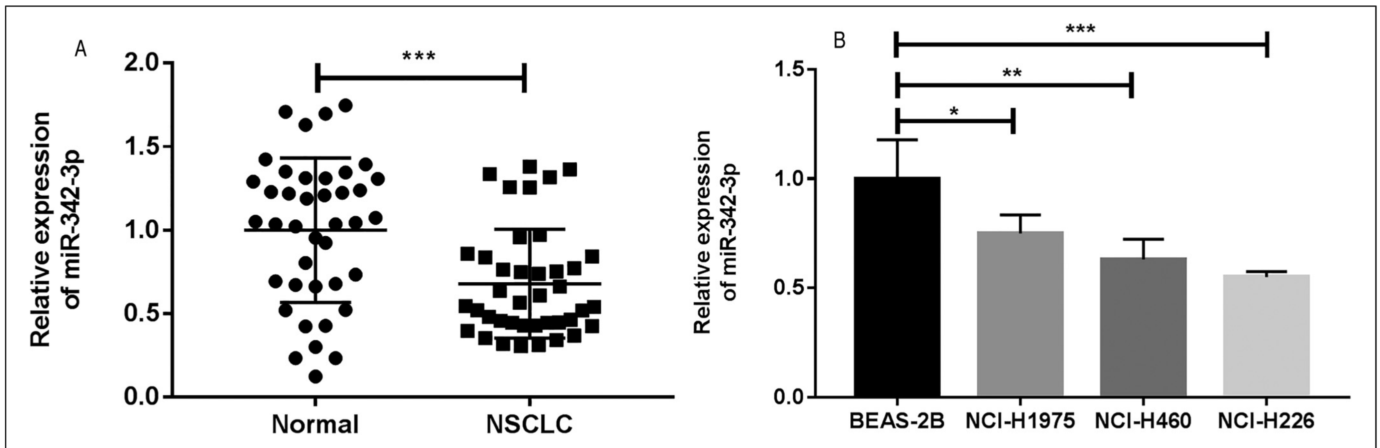

Figure 1. qRT-PCR analysis of the miRNA-342-3p expression levels in NSCLC tissues and cell lines. (A) The miRNA-342-3p expression levels in NSCLC cancer tissues (n = 40) and adjacent normal noncancerous tissues (n = 40) were measured by qRT-PCR. (B) The average relative expression levels of miRNA-342-3p were determined in 3 NSCLC cell lines (H1975, H460, and H226) and one normal cell line (BEAS-2B). Data are presented as mean ± SD of 3 independent experiments. Error bars represent SD.
Abbreviations: NSCLC, nonsmall cell lung cancer; qRT-PCR, quantitative reverse-transcription-polymerase chain reaction; miRNAs, MicroRNAs.

miR-NC cells (5%) (Figure 2E to G), indicating that the overexpression of miRNA-342-3p significantly promoted the apoptosis of H1975, H460, and H226 cells. These results indicate that miRNA-342-3p negatively regulates the growth of NSCLC cells.

BCL-2 is a Direct Target of miRNA-342-3p

Using the miRNA database, TargetScan, we identified *BCL-2* as a potential target gene of miRNA-342-3p. To confirm whether miR-133b regulates *BCL-2* expression by directly binding to the 3′-UTR of *BCL-2*, the WT or mutant BCL-2 (3′-UTR) was cloned into a vector downstream of the luciferase reporter gene and co-transfected with the miRNA-342-3p mimic or miRNA-342-3p inhibitor into HEK 293T cells (Figure 3A). miRNA-342-3p was found to significantly suppress the luciferase activity of wild-type *BCL-2* (3′-UTR-WT) but not the mutant *BCL-2* (3′-UTR-MUT) in HEK 293T cells (Figure 3B). These results suggest that miRNA-342-3p directly binds to putative *BCL-2* 3′-UTR regions, as predicted.

Table 1. The Association Between miR-342-3p Expression and Clinicopathological Features in NSCLC (n = 40).

Clinicopathological features	Number of cases (%)	miR-342-3p expression Low (20)	High (20)	Chi-Square test *P*-value
Age				0.3112
<60	13 (32.5)	8	5	
≥60	27 (67.5)	12	15	
Sex				0.5250
Male	18 (45)	10	8	
Female	22 (55)	10	12	
Differentiation degree				0.3272
High/moderate	15 (37.5)	6	9	
Poor	25 (62.5)	14	11	
Tumor Node Metastasis stage				0.8097
I	5 (12.5)	2	3	
II	10 (25)	4	6	
III	18 (45)	10	8	
IV	7 (17.5)	4	3	
Size (cm)				0.0044**
<3	21 (52.5)	6	15	
≥3	19 (47.5)	14	5	

Abbreviations: NSCLC, nonsmall cell lung cancer.

We proceeded to analyze the expression of *BCL-2* in NSCLC cells. The relative content of *BCL-2* mRNA in 80 samples was detected by qRT-PCR, which revealed that the *BCL-2* mRNA was markedly expressed in NSCLC tissues relative to the noncancerous controls (Figure 3D). To confirm this relationship, we further analyzed the relationship between *BCL-2* mRNA and miRNA-342-3p. As a result, we found a significant negative correlation between miRNA-342-3p and *BCL-2* ($P < .05$, Figure 3E). As a supplement, we detected significantly high mRNA and protein expression levels of *BCL-2* in H1975, H460, and H226 cells (Figure 3F to H).

BCL-2 Production is Negatively Regulated by miRNA-342-3p

The mRNA and protein expression levels of *BCL-2* in H1975, H460, and H226 cells the following co-transfection with the miRNA-342-3p mimic or miRNA-342-3p inhibitor were examined. According to qRT-PCR analysis, *BCL-2* mRNA expression was inhibited after transfection of the miRNA-342-3p mimic into H1975, H460, and H226 cells, whereas *BCL-2* mRNA expression was significantly enhanced after transfection with the miRNA-342-3p inhibitor (Figure 4A, C, and E). Similarly, BCL-2 protein levels were decreased or enhanced

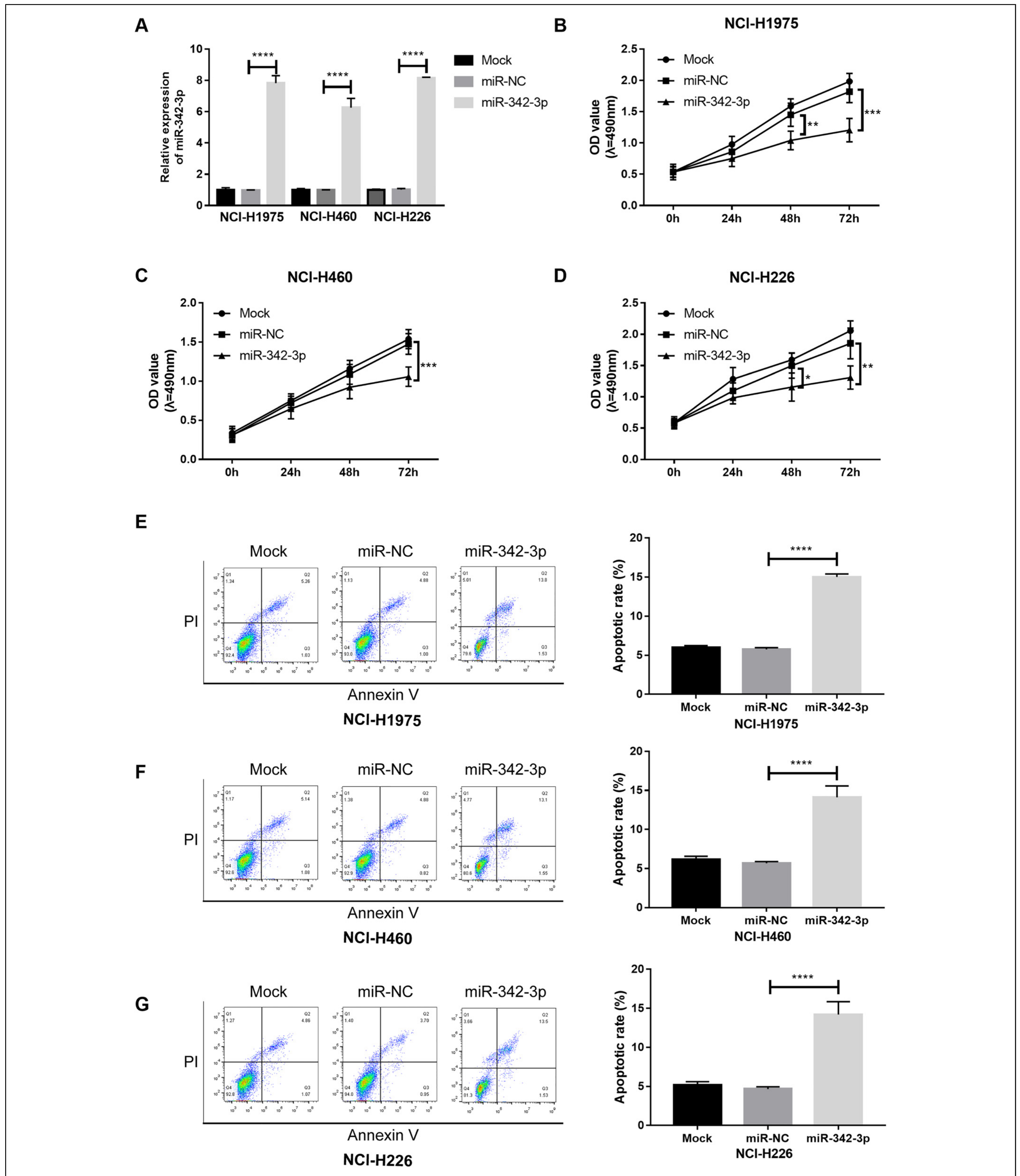

Figure 2. Effect of miRNA-342-3p on the proliferation and apoptosis of NSCLC cells. (A) The miRNA-342-3p expression levels in H1975, H460, or H226 cells transfected with miRNA-342-3p or miR-NC were examined by qRT-PCR. (B-D). The cell proliferative ability was compared between the miRNA-342-3p mimic and miR-NC transfected H1975, H460, or H226 cells using the CCK-8 assay (E-G). Flow cytometry was performed with H1975, H460, or H226 cells transfected with miRNA-342-3p or miR-NC to determine the statistics of apoptosis. Data are presented as mean ± SD of 3 independent experiments. Error bars represent SD.

Abbreviations: NSCLC, nonsmall cell lung cancer; qRT-PCR, quantitative reverse-transcription-polymerase chain reaction; miRNAs, MicroRNAs.

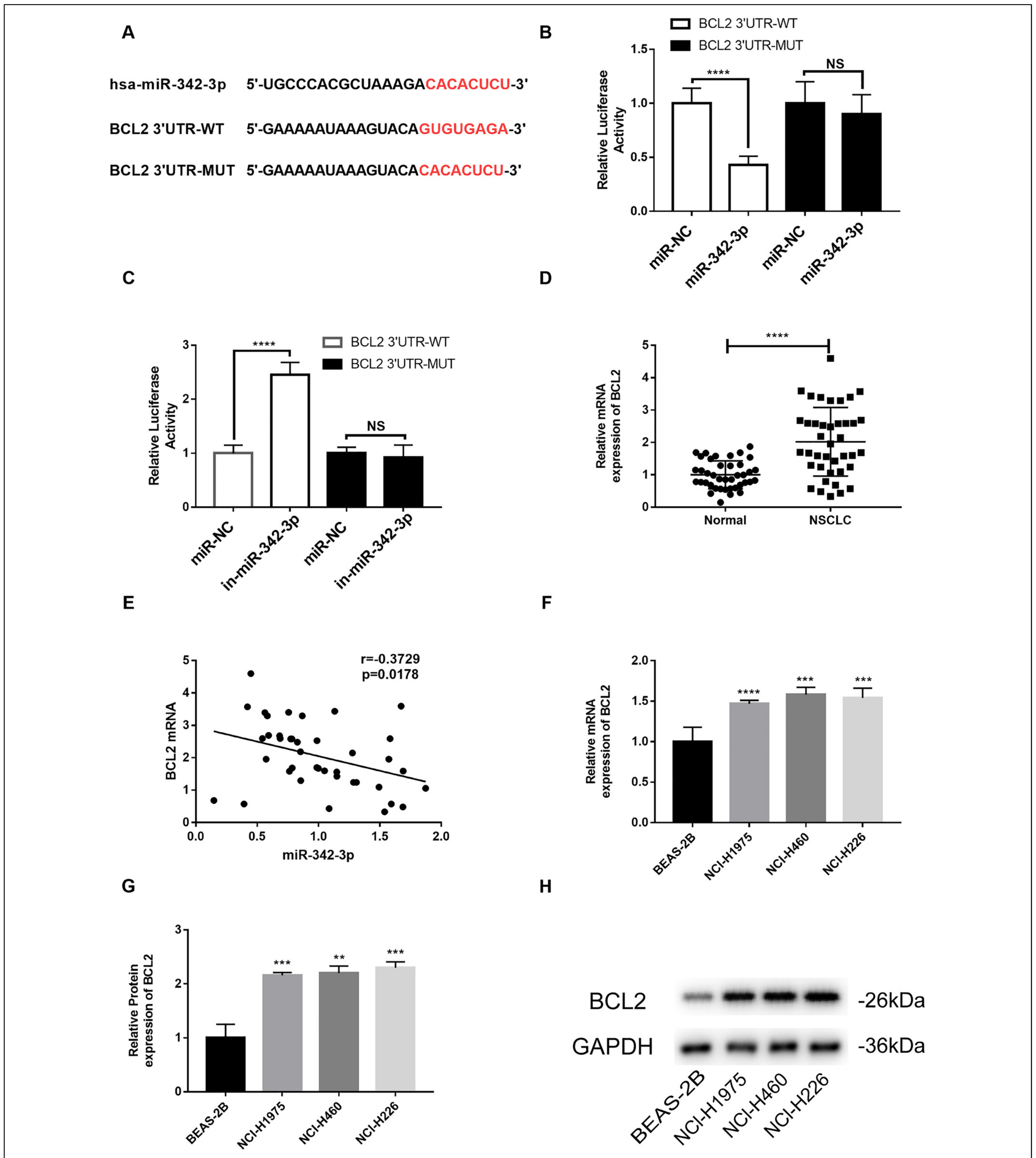

Figure 3. BCL-2 is a direct target of miRNA-342-3p and its expression in NSCLC tissues and cell lines. (A) Sequence alignment of human miRNA-342-3p with the 3′-UTR of BCL-2 mRNA. (B, C) The luciferase activities of the 3′-UTRs of wild-type BCL-2 (WT) and mutant BCL-2 (MUT) co-transfected with the miRNA-342-3p mimic, miRNA-342-3p inhibitor, or a negative control (miR-NC) were measured. (D) The expression levels of BCL-2 mRNA in 40 cancer tissues and 40 normal tissues were analyzed by qRT-PCR. (E) Inverse correlation of miRNA-342-3p expression with BCL-2 mRNA expression based on Pearson's correlation analysis. (F-H) The expression levels of mRNA and proteins of BCL-2 in NSCLC cells.

Abbreviations: NSCLC, nonsmall cell lung cancer; miRNAs, MicroRNAs; BCL-2, B-cell lymphoma-2; qRT-PCR, quantitative reverse-transcription-polymerase chain reaction.

in H1975, H460, and H226 cells transfected with miR-221 mimic or inhibitor, respectively (Figure 4B, D, and F). Such findings confirm that *BCL-2* expression is negatively regulated by miRNA-342-3p.

BCL-2 is Essential for NSCLC Cell Growth

Furthermore, the expression of *BCL-2* was silenced by siRNA transfection in H1975, H460, and H226 cells, and the results showed a high knockdown efficiency of *BCL-2* in the cells at the mRNA level (Figure 5A). Based on the CCK-8 assays, the proliferative abilities of H1975, H460, and H226 cells transfected with the siRNA targeting *BCL-2* were significantly decreased (Figure 5B to D), which were identical to the phenotypes resulting from miRNA-342-3p overexpression (Figure 2B to D). Cell apoptosis assays revealed that many H1975, H460, and H226 cells (approximately 4-fold higher than the normal) underwent apoptosis due to *BCL-2* knockdown (Figure 5E to G). Our data revealed that *BCL-2* might function as a tumor promoter in NSCLC by promoting cell proliferation and repressing cell apoptosis.

The miRNA-342-3p/BCL-2 Axis Regulates the Proliferation and Apoptosis of NSCLC Cells

We found that *BCL-2* was the target of miRNA-342-3p and promoted NSCLC progression. Thus, a rescue experiment was conducted to identify whether *BCL-2* overexpression could reverse the miRNA-342-3p overexpression phenotype. MiRNA-342-3p overexpression impaired the expression of *BCL-2* mRNA, whereas the co-overexpression of miRNA-342-3p and pcDNA-BCL-2 restored the *BCL-2* mRNA expression levels in H1975, H460, and H226 cells (Figure 6A, D, and G).

The CCK-8 assays suggested that miRNA-342-3p overexpression markedly inhibited the proliferative abilities of NSCLC cells, while miRNA-342-3p and pcDNA-BCL-2 co-overexpression restored them, further increasing the growth of the cells (Figure 6B, E, and H). The overexpression of miRNA-342-3p upregulated apoptosis. However, the overexpression of pcDNA-BCL-2 blocked the promotion of apoptosis induced by miRNA-342-3p in H1975, H460, and H226 cells (Figure 6C, F, and I). Altogether, these findings suggest that miRNA-342-3p suppresses NSCLC cell growth by directly targeting the expression of *BCL-2*.

Upregulation of miRNA-342-3p Suppresses Tumor Growth by Inhibiting BCL-2 In Vivo

Previous studies investigating the proliferative abilities of NSCLC cells were mainly carried out in vitro by transfecting miRNA-342-3p mimics. The tumor-suppressive effects of miRNA-342-3p were also demonstrated by its ability to inhibit tumor growth in vivo. We established subcutaneous tumor models in BALB/c nude mice using H1975, H460, and H226 cells. After 1 week, miRNA-342-3p mimics or miR-NC were directly injected into the implanted tumor and tail vein every 7 days until day 42. Tumor growth was found to be slower in these cells than in the control cells (Figure 7A). Further, the tumors appeared to be markedly lighter (Figure 7B). Compared to the tumors generated by the miR-NC group, we detected the upregulation of miRNA-342-3p and downregulation of *BCL-2* (Figure 7C and D). Similarly, decreased BCL-2 protein expression was observed in the miRNA-342-3p group (Figure 7E). Collectively, these findings demonstrate that miRNA-342-3p might suppress tumor growth by inhibiting *BCL-2* in vivo.

Discussion

MiRNAs have important roles in cancer diagnosis and treatment.[14–16] According to previous studies, miRNA-342-3p is often considered to be a tumor suppressor. For instance, miR-342-3p inhibits the migration and invasion of ovarian cancer cells by suppressing the expression of forkhead box Q1 (*FOXQ1*),[17] and suppresses the proliferation, colony formation, and invasion of nasopharyngeal cancer cells by targeting *FOXQ1*.[18] MiR-342-3p functions as a tumor suppressor in oral squamous cell carcinoma (OSCC) by targeting LIM and SH3 protein 1 (*LASP1*) and may act as a promising therapeutic target for OSCC.[19] In the present study, we found that miR-342-3p was downregulated in NSCLC tissues and cells, indicating that it may be a tumor suppressor in NSCLC, which is consistent with the above findings.

Considering the high mortality rates associated with lung cancer, there is an urgent need to identify novel biomarkers for the early diagnosis of the disease, which can also act as therapeutic targets for the treatment of lung cancer. The implementation of miRNAs as important regulators in NSCLC has been extensively evaluated, thereby increasing the methods available to manage lung cancer. The role of miR-342-3p has been extensively studied in many types of cancer. In fact, its role as an anti-oncogene is underlined in multiple studies reporting the downregulated expression of this sequence in patients diagnosed with this malignancy, including NSCLC.[20] The anticarcinogenic advantage generated by miR-342-3p in NSCLC is due to the involvement of target genes in multiple pathways, including cell growth, proliferation, angiogenesis, invasion, and metastasis as well as their resistance to chemotherapy and radiotherapy.[21] The therapeutic modulation of miR-342-3p using different targets entrapped in different delivery systems has shown promising results in the impairment of NSCLC. In NSCLC, miR-342-3p suppresses cell proliferation and migration by targeting anterior gradient 2 (*AGR2*),[22] and suppresses cell proliferation and invasion by targeting *RAS2B*.[23] Hence, further studies are necessary to elucidate the roles of miR-342-3p in NSCLC and the mechanisms of its associated pathways.

To better understand the underlying mechanism of miR-342-3p in NSCLC, we studied its biological functions in vitro and in vivo. In the present study, we showed that the upregulation of miR-342-3p inhibited the proliferation of H1975, H460, and H226 cells, while promoting apoptosis.

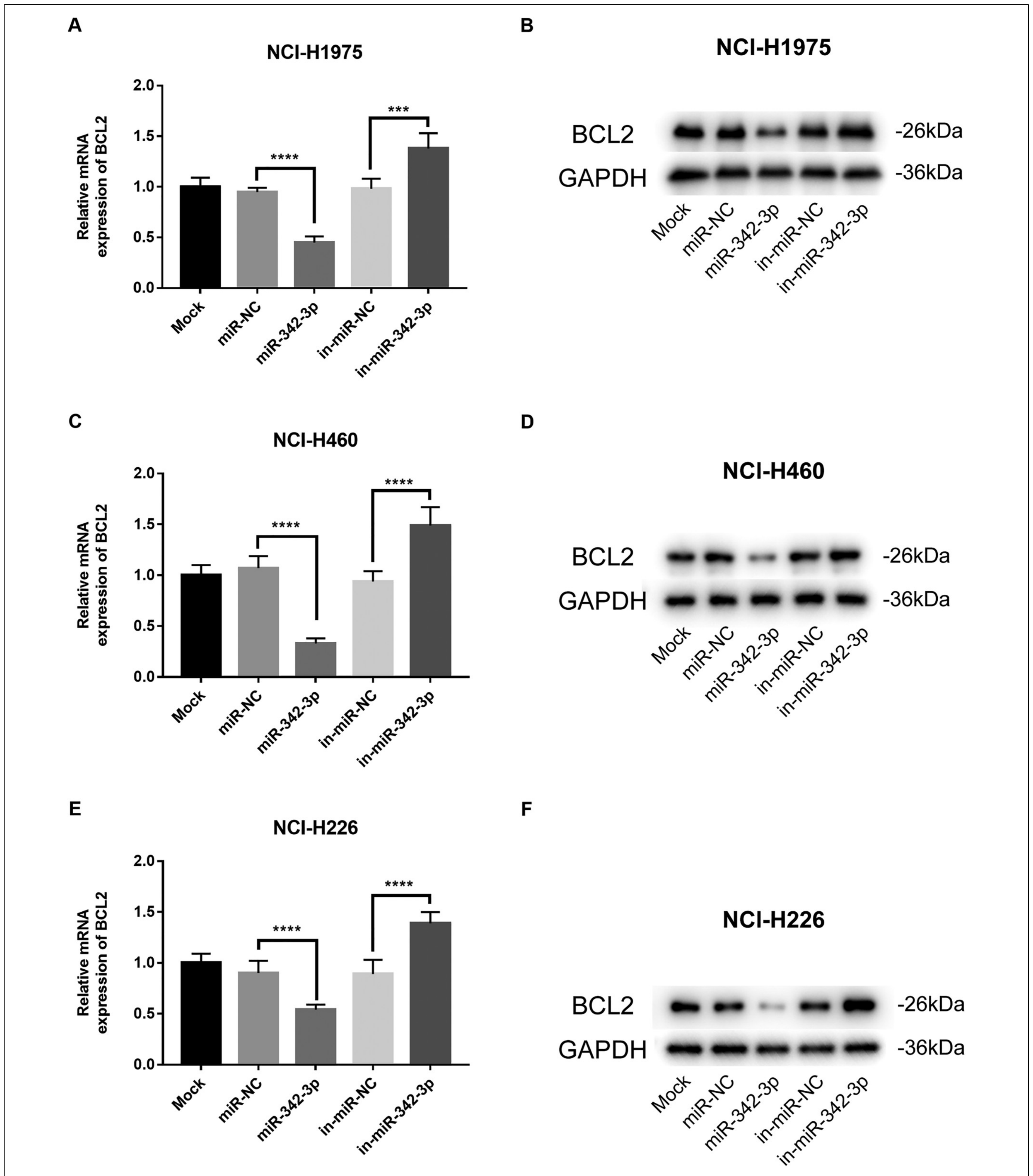

Figure 4. MiRNA-342-3p negatively regulated BCL-2 in the NSCLC cell lines. (A, C, and E) The relative mRNA levels of BCL-2 in H1975, H460, and H226 cells co-transfected with the miRNA-342-3p mimics, miRNA-342-3p inhibitor, or a negative control (miR-NC) were measured by qRT-PCR; (B, D, and F) The protein levels of BCL-2 in H1975, H460, and H226 cells co-transfected with the miRNA-342-3p mimic, miRNA-342-3p inhibitor, or a negative control (miR-NC) were determined by western blotting. β-actin was used as an internal control. Data are presented as mean ± SD of 3 independent experiments. Error bars represent SD.

Abbreviations: NSCLC, nonsmall cell lung cancer; miRNAs, MicroRNAs; BCL-2, B-cell lymphoma-2; qRT-PCR, quantitative reverse-transcription-polymerase chain reaction.

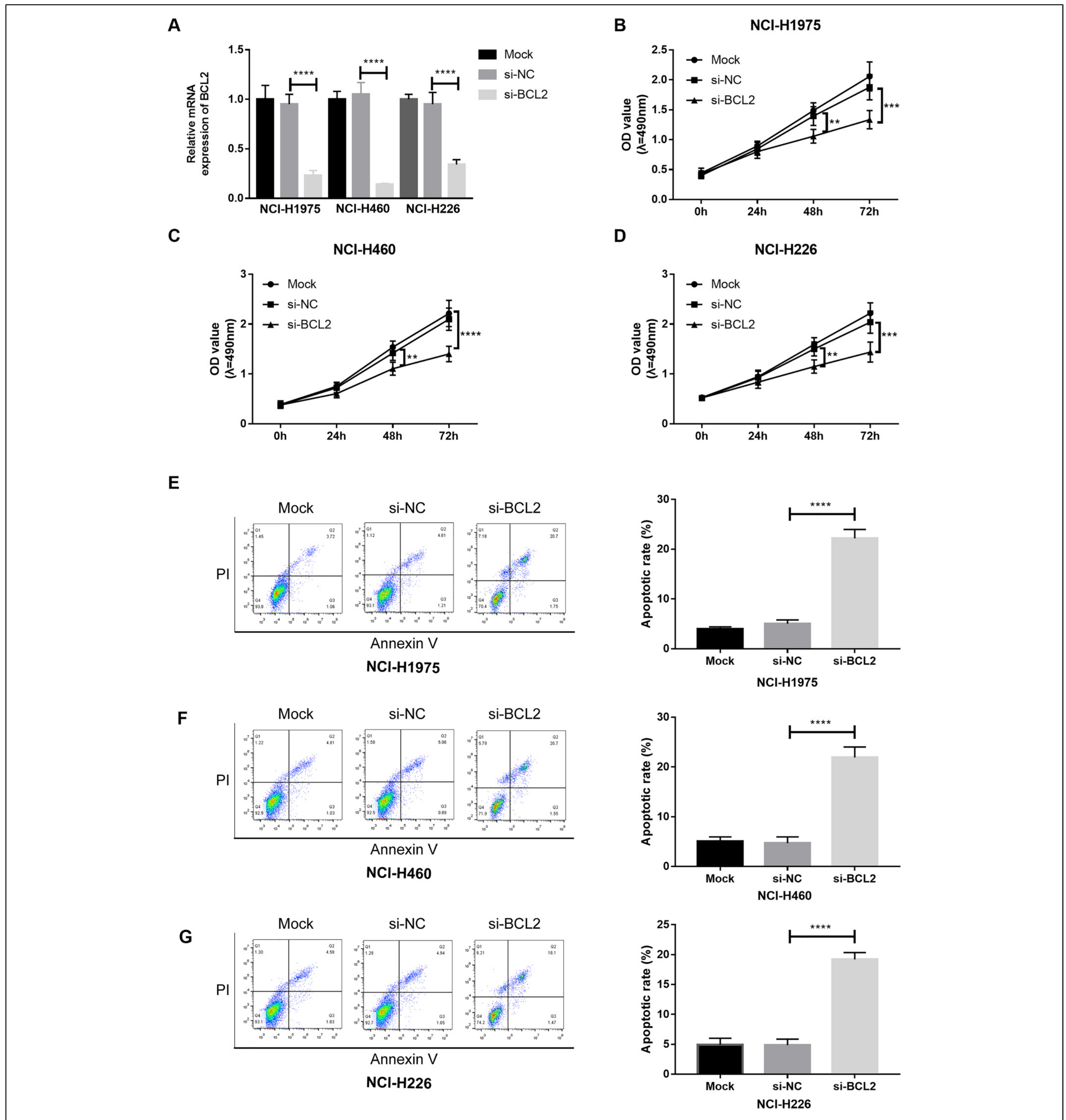

Figure 5. Effects of BCL-2 knockdown on the growth of NSCLC cells. (A) The expression of BCL-2 at the mRNA level post-siRNA silencing in NSCLC cells. (B-D) Cell proliferation was assessed in si-BCL-2 or si-NC transfected H1975, H460, and H226 cells using the CCK-8 assay; (E-G) Flow cytometry was performed in si-BCL-2 or si-NC transfected H1975, H460, and H226 cells to determine the statistics of apoptosis. Data are presented as mean ± SD of 3 independent experiments. Error bars represent SD.
Abbreviations: NSCLC, nonsmall cell lung cancer; miRNAs, MicroRNAs; BCL-2, B-cell lymphoma-2.

Moreover, the overexpression of miR-342-3p resulted in a significant decrease in tumor weight in a nude mice xenograft model. These results are consistent with previous findings and prove that miR-342-3p functions as a tumor suppressor in NSCLC.

As previously mentioned, a single miRNA usually modulates a signaling network by targeting the corresponding genes that have multiple functions. We found that *BCL-2* is a potential target of miR-342-3p. The BCL-2 family of proteins

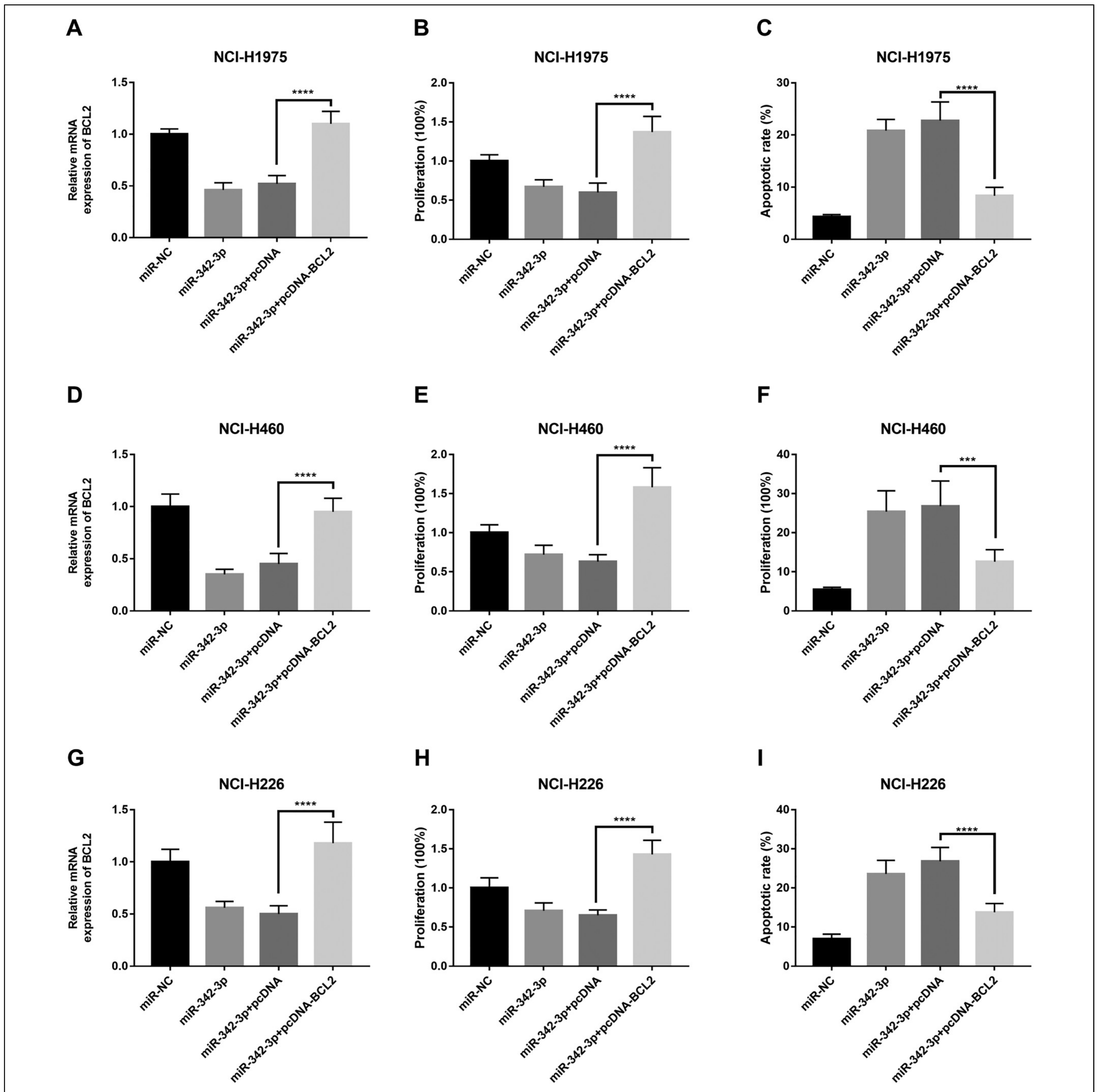

Figure 6. Rescue experiment to verify the relationship between miRNA-342-3p and BCL-2 in NSCLC cells. (A, D, and G) The relative expression levels of BCL-2 mRNA were measured in miRNA-342-3p- or miR-NC-overexpressing H1975, H460, and H226 cells with/without pcDNA, or with pcDNA-BCL-2 overexpression by qRT-PCR; (B, E, and H) CCK-8 assays were used to determine the proliferation in the above cell groups; (C, F, and I) Flow cytometry was performed to determine the statistics of apoptosis of the above cell groups. Data are presented as mean ± SD of 3 independent experiments. Error bars represent SD.

Abbreviations: NSCLC, nonsmall cell lung cancer; miRNAs, MicroRNAs; BCL-2, B-cell lymphoma-2; qRT-PCR, quantitative reverse-transcription-polymerase chain reaction.

acts as key regulators of apoptosis, and *BCL-2* dysfunctions have been implicated in many diseases, including ischemia, neurodegenerative disorders, autoimmune diseases, and cancer.[24–26] *BCL-2* expression has been linked to multiple cancers, including prostate, lung, ovary, and breast cancer.[27] When *BCL-2* expression was suppressed in NSCLC, cell proliferation was inhibited, while apoptosis was promoted. *BCL-2* is a target of several miRNAs, including miR-17 to 92, miR-15,

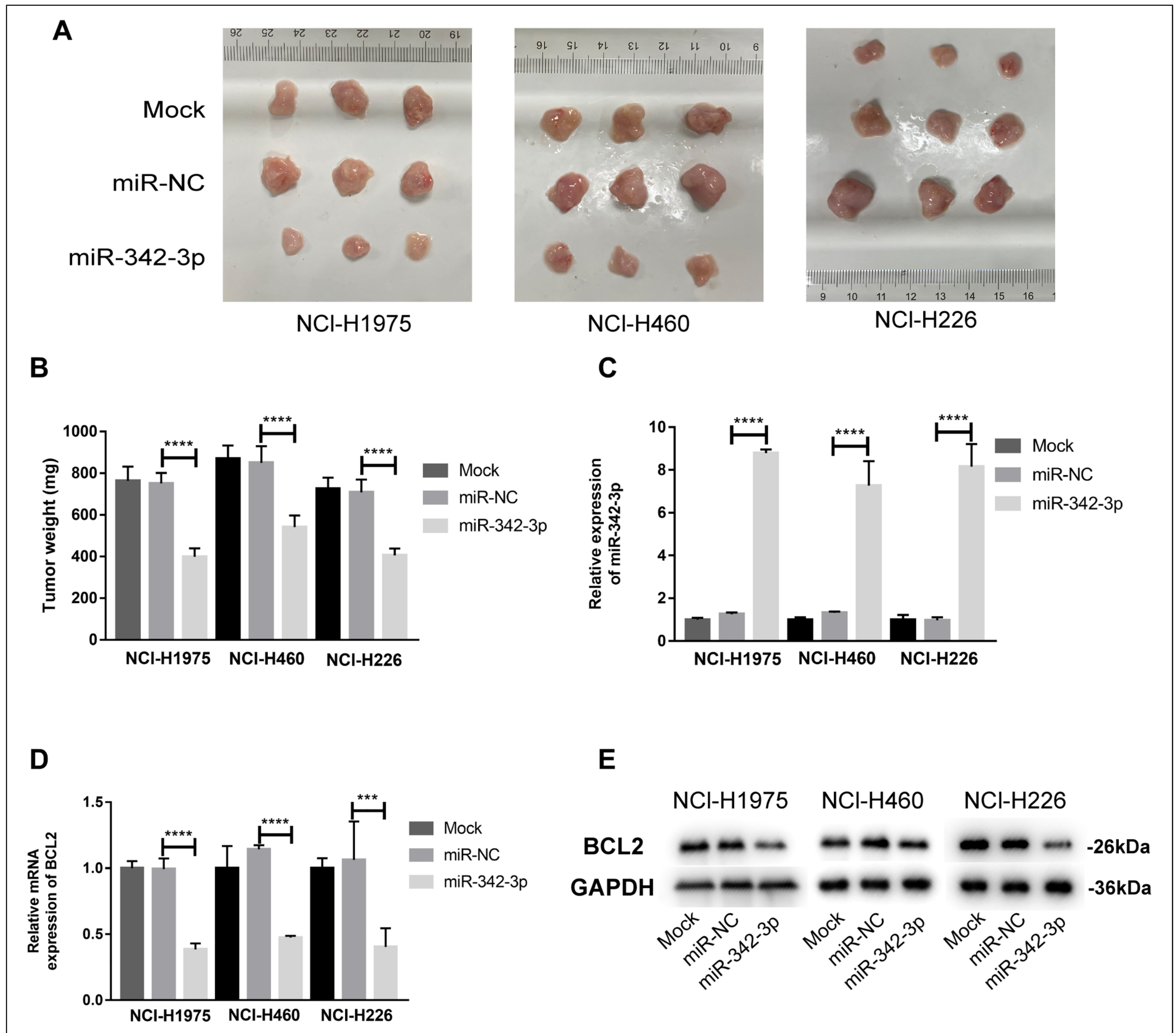

Figure 7. MiRNA-342-3p suppressed tumors in vivo. The tumor (A) sizes and (B) weights of every mice model were measured. Three mice models were involved in each group. (C) The relative expression of miRNA-342-3p in NSCLC tumor was measured. (D, E) The relative expression levels of mRNA and proteins of BCL-2 in NSCLC tumor were measured. Flow cytometry was performed to determine the statistics of apoptosis of the above cell groups. Data are presented as mean ± SD of 3 independent experiments. Error bars represent SD.
Abbreviations: NSCLC, nonsmall cell lung cancer; miRNAs, MicroRNAs; BCL-2, B-cell lymphoma-2.

and miR-16.[28–30] In this study, the luciferase reporter assays revealed that BCL-2 may act as the direct target of miR-342-3p. qRT-PCR and western blotting analyses further confirmed that BCL-2 expression was significantly decreased in NSCLC cells transfected with the miR-342-3p mimic, and significantly increased in cells transfected with the miR-342-3p inhibitor, compared to the corresponding negative control. These results suggest that *BCL-2* is a target of miR-342-3p in NSCLC cells.

In conclusion, our results showed that miR-342-3p is down-regulated in NSCLC and regulates the proliferation and apoptosis of NSCLC cells by targeting *BCL-2*. Thus, miR-342-3p may be used to design new approaches for NSCLC treatment by harnessing the mechanisms employed to regulate tumor growth.

Authors' Contributions

Wang HF contributed to the study concept and design; Chen ZJ performed the main experiments and wrote and revised the manuscript; Ying JJ, Shang WJ, Ding DX, and Guo M partly contributed to the experiments, data analysis, and interpretation. All authors read and approved the final manuscript.

Research Ethics and Patient Consent

The present study was approved by the Ethics Committee of Zhejiang University School of Medicine, First Affiliated Hospital, Beilun Branch. Informed consent was obtained from all patients before the start of this study.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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