

Lentivirus-mediated gene silencing of *NOB1* suppresses non-small cell lung cancer cell proliferation

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Abstract. NIN/RPN12 binding protein 1 (NOB1p) encoded by *NOB1* has been found to be an essential factor in 26S proteasome biogenesis which participates in protein degradation. However, the functions of *NOB1* in non-small cell lung cancer cells are largely unknown. In the present study, lentivirus-mediated *NOB1* shRNA transfection in two non-small cell lung cancer cell lines (A549 and H1299) was accomplished, as determined by fluorescence imaging. Downregulation of *NOB1* expression was confirmed by real-time PCR and western blotting. *NOB1* silencing resulted in a significant decline in the proliferation and colony formation capability of non-small cell lung cancer cells. Moreover, flow cytometry showed that A549 cells were arrested in the G0/G1 phase of the cell cycle after *NOB1* suppression. Furthermore, depletion of NOB1 resulted in a significant decrease in CDK4 and cyclin D1 expression. These results suggest that *NOB1* may act as an important regulator in non-small cell lung cancer growth and could be a therapeutic target of non-small cell lung cancer.

Introduction

Lung cancer is one of the leading causes of cancer-related death worldwide, and has the highest cancer mortality rate among all cancers in males worldwide and the highest mortality rate among females in developing countries (1,2). Non-small

cell lung cancer is the most common type of lung cancer. Therefore, effective treatments for preventing non-small cell lung cancer and promoting the survival rate of patients have been extensively investigated. As multiple genetic alterations are involved in a chronic process that leads to cancers, it could be suggested that the regulation of non-small cell lung cancer-associated genes may contribute to non-small cell lung cancer therapy. RNA interference (RNAi)-mediated cancer therapy whose function is to downregulate relevant transcripts has been identified as a novel and effective process in regards to therapeutic strategy (3). To date, numerous genes influencing non-small cell lung cancer cells have been confirmed, such as nuclear factor of activated T cells (NFAT) (4), nuclear factor, erythroid-derived 2, like 2 (NRF2) (5) and metastasis-associated protein 1 (MTA1) (6). These developments herald a promising future for gene-targeted therapy for non-small cell lung cancer.

NIN/RPN12 binding protein 1 (NOB1p) encoded by *NOB1* was first identified in *Saccharomyces cerevisiae* by the two-hybrid screening method as a binding protein that interacts with Nin1p/Rpn12p which is a subunit of 19S regulatory particle of the yeast 26S proteasome (7). NOB1p joins the 20S proteasome with the 19S regulatory particle and promotes the maturation of the 20S proteasome, and then NOB1p is internalized into the 26S proteasome and degraded to complete 26S proteasome biogenesis in eukaryotes (8). Additionally, as a ribosome assembly factor, NOB1p is essential for processing of the 20S pre-rRNA to the mature 18S rRNA (9,10). NOB1p also serves as a part of a pre-40S ribosomal particle that is transported from the nucleus to the cytoplasm and consequently cleaves site D at the 3' end of mature 18S rRNA (9,10). This evolutionarily conserved protein contains a PIN domain which is required for pre-rRNA cleavage, RNAi process and nonsense-mediated mRNA decay (9,11). The human *NOB1* is located on human chromosome 16q22.1 and includes nine exons. The length of the cDNA sequence is 1,749 bp and contains an open reading frame 1,239-bp long. The *NOB1* mRNA is mainly expressed in the liver, lung and spleen, and is localized in the nucleus (12). Furthermore, the expression of NOB1p in papillary thyroid carcinoma cells (13) and breast

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cancer cells (14) is significantly higher than that in normal tissue cells.

In the past few years, many efforts have been made to indicate the role of *NOBI* in tumor development. Thus, in the present study, to investigate the biological function of *NOBI* in non-small cell lung cancer, we employed lentivirus-mediated short hairpin RNA (shRNA) to silence *NOBI* expression in two established non-small cell lung cancer cell lines. Then the effects of *NOBI* knockdown on the proliferation, colony formation and cell cycle progression of non-small cell lung cancer cells were studied.

Materials and methods

Reagents and plasmids. Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 medium were obtained from Hyclone (Beijing, China). Opti-MEM medium and fetal calf serum (FCS) were obtained from Gibco (Cambrex, USA). Lipofectamine 2000 and TRIzol were purchased from Invitrogen (Carlsbad, CA, USA). Isopropanol and crystal violet were obtained from Sinopharm Chemical Reagent Co., Ltd., and Beyotime Institute of Biotechnology, respectively. All other reagents were purchased from Sigma (St. Louis, MO, USA). pFH-L, pCMVΔR8.92 and pVSVG-I plasmids as well as helper plasmids (pHelper 1.0 and pHelper 2.0) were purchased from Hollybio (Shanghai, China).

Immunohistochemistry (IHC). Twenty-nine non-small cell lung cancer specimens were collected for immunohistochemistry (15 males, 14 females; 16 specimens from patients younger than 60 years of age, 13 from patients older than 60 years of age). Ten normal lung specimens were used as control. All of the above tissue samples were provided by the Department of Thoracic Surgery at the First Hospital of Shanghai, and patients provided informed consent forms conforming to the guidelines of the Declaration of Helsinki. The tissues were fixed with formalin and embedded in paraffin. Before immunostaining, the paraffin was removed from the samples. Samples were blocked and incubated with the primary antibody against NOBI (cat no. GTX120935, dilution 1:100; GeneTex) overnight at 4°C and a biotinylated secondary antibody for 30 min at room temperature. After reacting with streptavidin-peroxidase conjugate for 10 min at room temperature, DAB staining was performed. All samples were counterstained with hematoxylin.

Cell culture. Human embryonic kidney cell line 293T (HEK293T) and human non-small cell lung cancer cell lines A549 and H1299 were obtained from the Cell Bank of the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). HEK293T and A549 cells were cultured in DMEM supplemented with 10% FBS at 37°C in a 5% CO₂ humidified incubator. H1299 cells were cultured in RPMI-1640 supplemented with 10% FBS at 37°C in a 5% CO₂ humidified incubator.

Construction of recombinant lentivirus. shRNA for the human *NOBI* gene (NM_020143) and the non-silencing control shRNA were: 5'-CTAGCCCGGTTCTCCGAACGTGTCACGTATCTCGAGATACGTGACACGTTCCGGAGAATTTTT

TAAT-3' and 5'-CTAGCCCGGCCAAGGAAGTGCAATTGCATACTCGAGTATGCAATTGCACTTCCTTGGTTTTTGTTAAT-3', respectively. Subsequently, they were inserted into the lentiviral vector pFH-L. HEK293T cells were cultured in 10-cm dishes at the concentration of 6x10⁵ cells/ml for 24 h. Two hours before transfection, the medium was replaced by serum-free DMEM. The modified pFH-L plasmids, lentiviral packing vector pCMVΔR8.92, pVSVG-I plasmids and helper plasmids (pHelper 1.0 and pHelper 2.0) were transected into 70-80% confluent HEK293T cells via Lipofectamine 2000 to generate the recombinant lentivirus. After incubation for 48 h, the lentivirus was harvested, collected and concentrated by Centricon-Plus-20 filter devices (Millipore, USA).

Lentivirus-mediated infection in non-small cell lung cancer cells. Lentivirus-mediated *NOBI* and non-silencing control shRNA were infected into A549 and H1299 cells and seeded into 6-well plates at a density of 5x10⁴ cells/well by replacing the medium with Opti-MEM medium containing Polybrene (5 μg/ml). After 48 h, the medium was replaced with fresh medium and incubated for another 48 h. Then the cells were examined under a fluorescence microscope (CKX41; Olympus, Japan) by observing the green fluorescence emitted by the green fluorescent protein (GFP) in the lentivirus particles.

RNA extraction and real-time PCR. A549 and H1299 cells were cultured in 6-well plates and infected with recombinant lentivirus for 6 days. The cells were lysed with TRIzol reagent and total RNA was isolated from the lysate. The cDNA was synthesized from total RNA (2 μg) using Promega M-MLV cDNA Synthesis kit according to the manufacturer's instructions. *NOBI* mRNA expression was determined by real-time PCR (CFX96; Bio-Rad, USA) using SYBR-Green PCR core reagents with Bio-Rad Connect Real-Time PCR platform. For *NOBI* detection, forward, 5'-GAAAGAACAACGCCCTGGAG-3' and reverse, 5'-CAGCCTTGAGATGACCTAAGC-3' were designed, respectively. Parallel reactions were performed using primers (forward, 5'-GTGGACATCCGCAAAGAC-3' and reverse, 5'-AAAGGGTGTAAACGCAACTA-3') for actin as an internal control. The relative *NOBI* mRNA expression level as compared with actin was evaluated using the 2^{-ΔΔC_t} analysis method.

Western blot analysis. A549 and H1299 cells were cultured in 6-well plates and infected with the recombinant lentivirus for 6 days. The cells were then washed twice with ice-cold PBS and lysed in 2X SDS sample buffer (10 mM EDTA, 4% SDS, 10% glycine in 100 mM Tris-HCl buffer, pH 6.8) for 1 h at 4°C. Total cell lysates were then centrifuged (12,000 rpm, 15 min, 4°C), and the supernatants were employed for further processing. The protein concentration was determined by using the BCA protein assay kit. Equal amounts of proteins (30 μg) were loaded and separated on 10% SDS-PAGE gels and transferred onto PVDF membranes (Millipore). Proteins were probed overnight at 4°C with primary antibodies: anti-NOBI (1:5,000, cat no. GTX120935; GeneTex), anti-CDK4 (1:500, #2906; Cell Signaling Technology), anti-cyclin D1 (CCND1) (1:1,000, cat no. 60186-1-Ig; Proteintech Group, Inc.), anti-CDKN2B (1:1,000, #4822; Cell Signaling

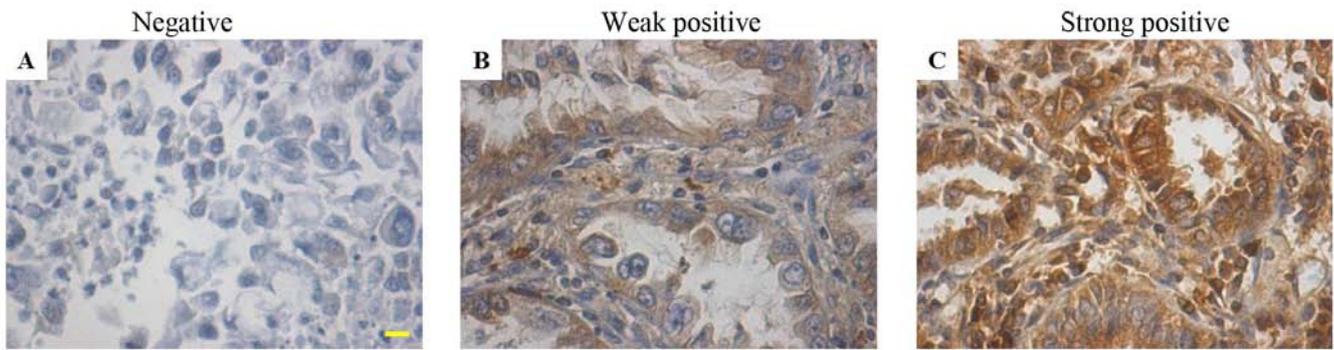


Figure 1. Immunohistochemical analysis of NOB1 expression in lung specimens. (A) Negative, (B) weak positive and (C) strong positive expression of NOB1 immunostaining in non-small cell lung cancer specimens.

Table I. Expression of NOB1 in the lung carcinoma specimens.

| Sample | N | NOB1 immunostaining | | | | Chi-square | P-value ^a |
|----------------|----|-----------------------|---------------------------------|-----------------------|------------------------------|------------|----------------------|
| | | Negative (-) n (%) | Weak positive (- to +) n (%) | Positive (+) n (%) | Hadro-positive (++) n (%) | | |
| Lung cancer | 29 | 2 (6.9) | 2 (6.9) | 21 (72.4) | 4 (13.8) | 21.03 | 0.0001 |
| Normal tissues | 10 | 8 (80.0) | 0 (0.0) | 2 (20.0) | 0 (0.0) | | |

^aUsing Pearson Chi-square test.

Technology), anti-CDKN2A (1:3,000, cat no. 10883-1-AP; Proteintech Group, Inc.) and anti-GAPDH (1:80,000, cat no. 10494-1-AP; Proteintech Group, Inc.), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000, cat no. SC-2004; Santa Cruz) at room temperature for 1 h. ECL reaction was performed using enhanced chemiluminescence (Amersham). Experiments were repeated at least three times.

Cell proliferation assay. Briefly, non-small cell lung cancer cells infected with *NOB1* shRNA lentivirus (Lv-shNOB1) or non-silencing shRNA lentivirus (Lv-shCon), and non-infected cells (Con) were seeded in a 96-well plate at an initial density of 2×10^3 cells/well. At specified incubation time-points, 20 μ l methylthiazol tetrazolium (MTT) solution (5 mg/ml) was added to each well. Following incubation at 37°C for 4 h, 100 μ l acidified isopropanol was added to the well to terminate the reaction, and then the samples were measured by a microplate reader (BioTek Epoch; BioTek, USA) at 595 nm.

Colony formation assay. A549 cells from different groups were seeded into 6-well plates at a density of 200 cells/well. The cells were cultured for 9 days to form colonies with the medium changed every 2 days. Then the cells were washed twice with PBS and fixed with 700 μ l 4% paraformaldehyde for 10 min. After being washed twice with PBS to remove the paraformaldehyde, the cells were stained with 700 μ l Giemsa solution for 5 min, and rinsed with PBS three times. The number of colonies containing >50 cells was counted under a microscope (CKX41; Olympus, Japan).

Cell cycle analysis. After infection for 4 days, A549 cells were seeded in 6-cm dishes at a density of 5×10^4 cells/dish. After the density reached ~80%, the A549 cells were collected, re-suspended in cold PBS and fixed with pre-cold 75% ethanol for 30 min at 4°C. Samples were washed with PBS and incubated with PBS containing RNAase and propidium iodide (PI) at 4°C overnight in the dark. Cell cycle progression was monitored using a flow cytometer (Beckman Coulter, Miami, FL, USA). Experiments were repeated at least three times.

Statistical analysis. The data are presented as mean \pm SD from at least three independent experiments. Statistical analysis was performed using the Student's t-test and SPSS 17.0 software, and $P < 0.05$ was considered to indicate a statistically significant result.

Results

***NOB1* is highly expressed in non-small cell lung cancer.** Previous studies have indicated that the expression of NOB1 is involved in several types of carcinomas. To investigate the function of NOB1 in non-small cell lung cancer, we evaluated the expression in 29 non-small cell lung cancer specimens using immunohistochemical staining. Of the 29 non-small cell lung cancer samples, 4 (13.7%) were hadro-positive, 21 (72.4%) were positive and 2 (6.9%) were weak positive, which was significantly higher than the levels in the normal lung tissue samples [none were hadro-positive, 2 out of 10 (20.0%) were positive and 8 out of 10 (80.0%) were negative] (Fig. 1 and Table I). These results suggest that

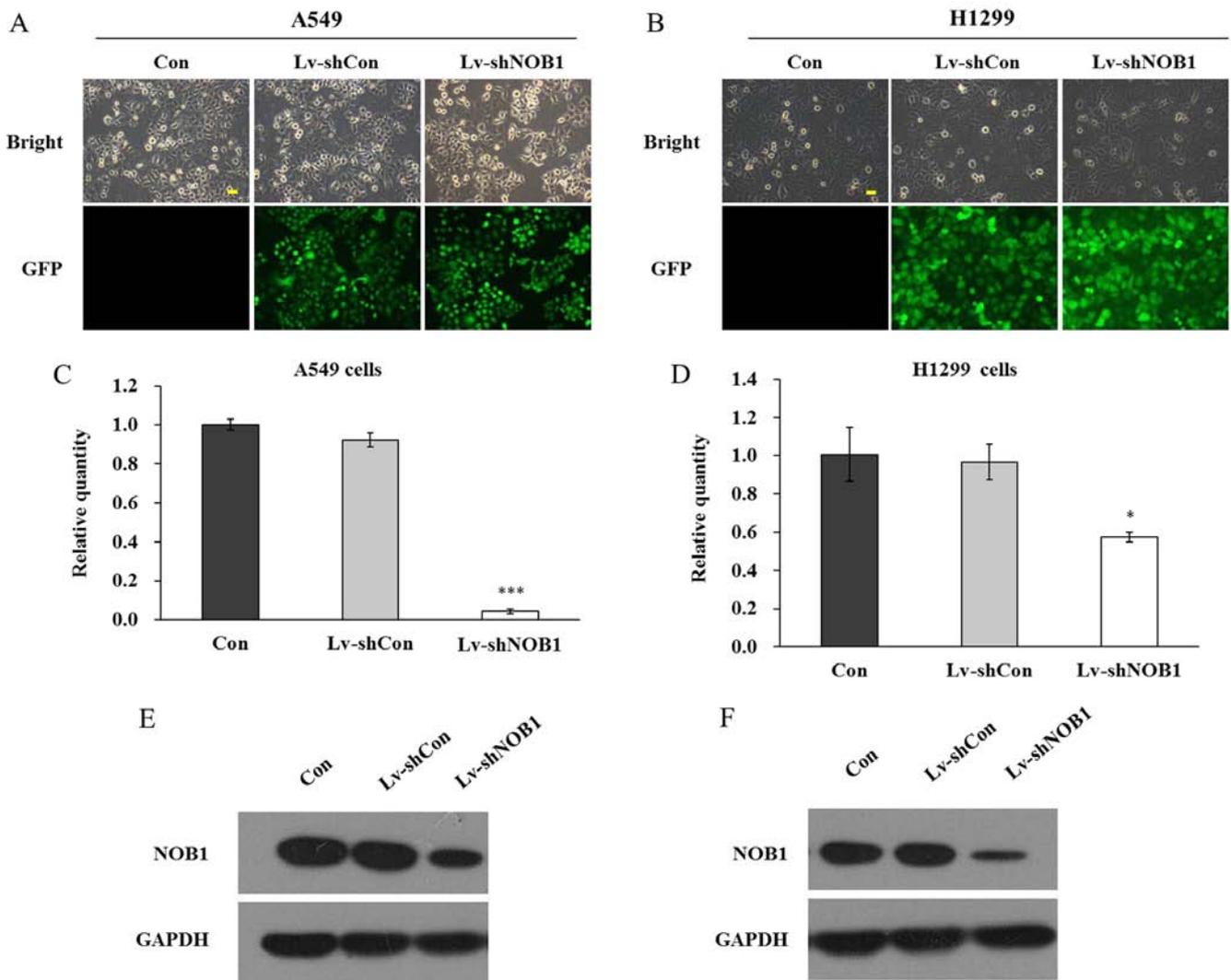


Figure 2. RNAi of NOB1 expression mediated by lentivirus. Representative images of A549 (A) and H1299 cells (B) infected with the recombinant lentivirus. NOB1 knockdown efficiency was measured with real-time PCR in the A549 (C) and H1299 (D) cells of the different groups. NOB1 knockdown efficiency was determined by western blotting in the A549 (E) and H1299 (F) cells of the different groups. * $P < 0.05$, *** $P < 0.001$ vs. the Lv-shCon group. RNAi, RNA interference. Lv-shCon, non-silencing shRNA lentivirus.

NOB1 is highly expressed in non-small cell lung cancer. The high expression level of NOB1 in non-small cell lung cancer suggests that it may be involved in the pathogenesis of non-small cell lung cancers.

Lentivirus-mediated RNAi inhibits NOB1 expression. Herein, NOB1 shRNA targets were cloned into the recombinant lentivirus plasmid, which was utilized to infect two established non-small cell lung cancer cell lines A549 and H1299. The non-silencing sequences were also inserted into the vector as a control. GFP fluorescence imaging was used to indicate the lentivirus infection as GFP was transfected into the cancer cells together with NOB1 shRNA. As shown in Fig. 2A and B, >80% cells were infected by the lentivirus as assessed by GFP fluorescence, indicating the successful transfection in both A549 and H1299 cells. NOB1 knockdown efficiency was determined by real-time PCR and western blotting. Lentivirus-mediated RNAi markedly decreased endogenous NOB1 mRNA expression, by 95.4% in the A549 cells and

40.7% in the H1299 cells (Fig. 2C and D). The protein level of NOB1 was also significantly reduced in both cell lines after lentivirus infection (Fig. 2E and F). Hence, lentivirus infection was confirmed to be effective to inhibit the expression of NOB1 in non-small cell lung cancer cells.

NOB1 knockdown inhibits non-small cell lung cancer cell proliferation. The effect of the silencing of NOB1 on non-small cell lung cancer cell proliferation was examined by MTT assay. As shown in Fig. 3, there was no significant difference in cell viability between the Lv-shCon infected and uninfected cells, suggesting no cytotoxic effect of the lentiviral system on both cell lines. Whereas, the proliferation rates of NOB1-silenced A549 cells and H1299 cells were significantly reduced as compared with the control groups from day 3. On day 5, the cell viability was decreased by 74.9% in the A549 cells and 27.5% in the H1299 cells after lentivirus infection, respectively. The inhibitory rate in the A549 cells was higher than the rate in the H1299 cells, consistent with the suppression

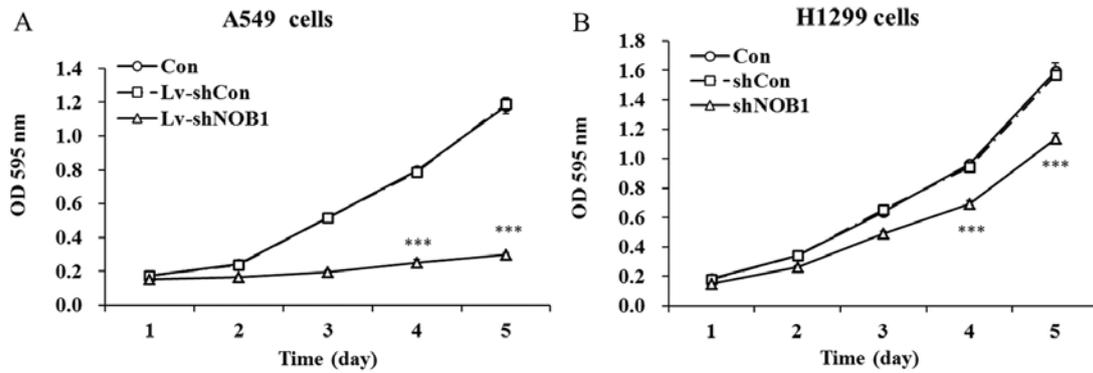


Figure 3. Effect of NOB1 knockdown on the proliferation of non-small cell lung cancer cells. MTT analysis of the proliferation rates of A549 (A) and H1299 (B) cells following lentivirus infection. *** $P < 0.001$ vs. the Lv-shCon group. Lv-shCon, non-silencing shRNA lentivirus.

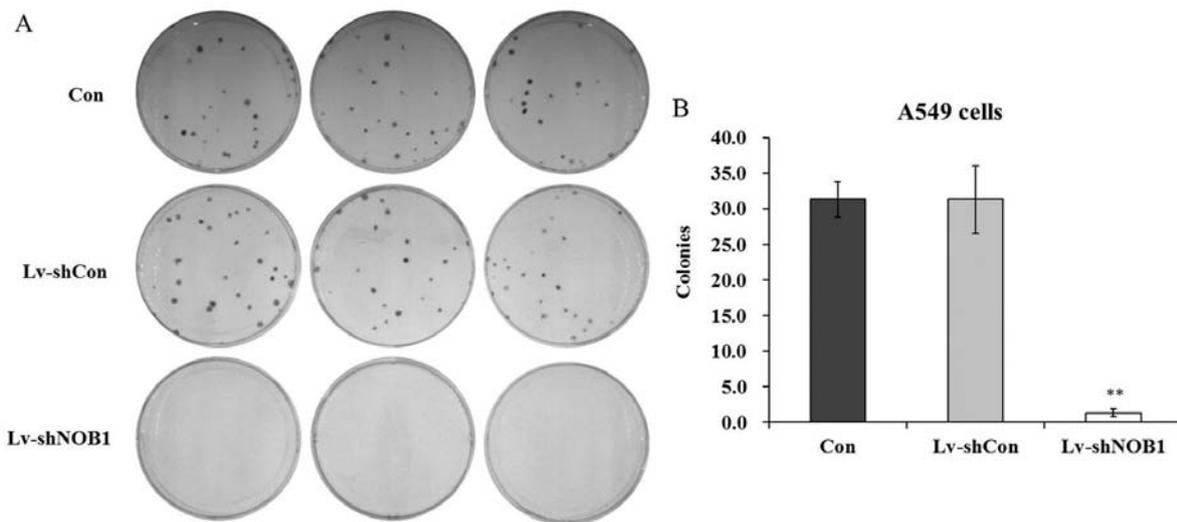


Figure 4. Effect of NOB1 knockdown on the colony formation of A549 cells. (A) Representative images of colonies formed in the A549 cells after infection for 9 days. (B) The average number of colonies of the A549 cells in the different groups. ** $P < 0.01$ vs. the Lv-shCon group. Lv-shCon, non-silencing shRNA lentivirus.

of *NOB1* expression by Lv-shNOB1. These results revealed the important functional role of *NOB1* in the proliferation of non-small cell lung cancer cells, and its inhibitory effect was dependent on the specific cell line.

***NOB1* depletion suppresses non-small cell lung cancer cell colony formation.** The colony formation assay by Giemsa staining was performed to evaluate the effect of *NOB1* depletion on the colony forming capability of A549 cells. Three groups of A549 cells (Con, Lv-shCon and Lv-shNOB1) were cultured for 9 days. The number of colonies in the Lv-shNOB1-infected A549 cells was markedly reduced by 96.8% as compared with the control groups as observed under a light microscope (Fig. 4A and B). The results indicated that knockdown of *NOB1* could also inhibit the colony formation of non-small cell lung cancer cells, representing its oncogenicity *in vitro*.

***NOB1* suppression leads to G0/G1 cell cycle arrest.** To ascertain the underlying mechanisms involved in the cell growth

inhibition induced following *NOB1* silencing, we analyzed the cell cycle distribution of the A549 cells after lentivirus infection (Fig. 5A). As shown in Fig. 5B, a higher percentage of cells was accumulated in the G0/G1 phase of the cell cycle ($69.32 \pm 0.45\%$) after Lv-shNOB1 infection, compared with the percentage in the Con group ($57.05 \pm 0.37\%$) and Lv-shCon group ($59.21 \pm 0.86\%$). The percentages of cells in the S phase and G2/M phase were markedly decreased after lentivirus infection. These results suggest that knockdown of *NOB1* suppressed the growth of non-small cell lung cancer cells possibly via induction of cell cycle arrest. Furthermore, alterations in the expression of cell cycle markers were detected in the A549 cells, including cyclin D1, CDK4, CDKN2A, and CDKN2B. Western blotting showed that depletion of *NOB1* resulted in a significant decrease in cyclin D1 and CDK4 expression, while no significant change was observed in CDKN2A and CDKN2B expression (Fig. 6). These results suggest that knockdown of *NOB1* in non-small cell lung cancer cells blocks cell cycle progression via downregulation of cyclin D1 and CDK4.

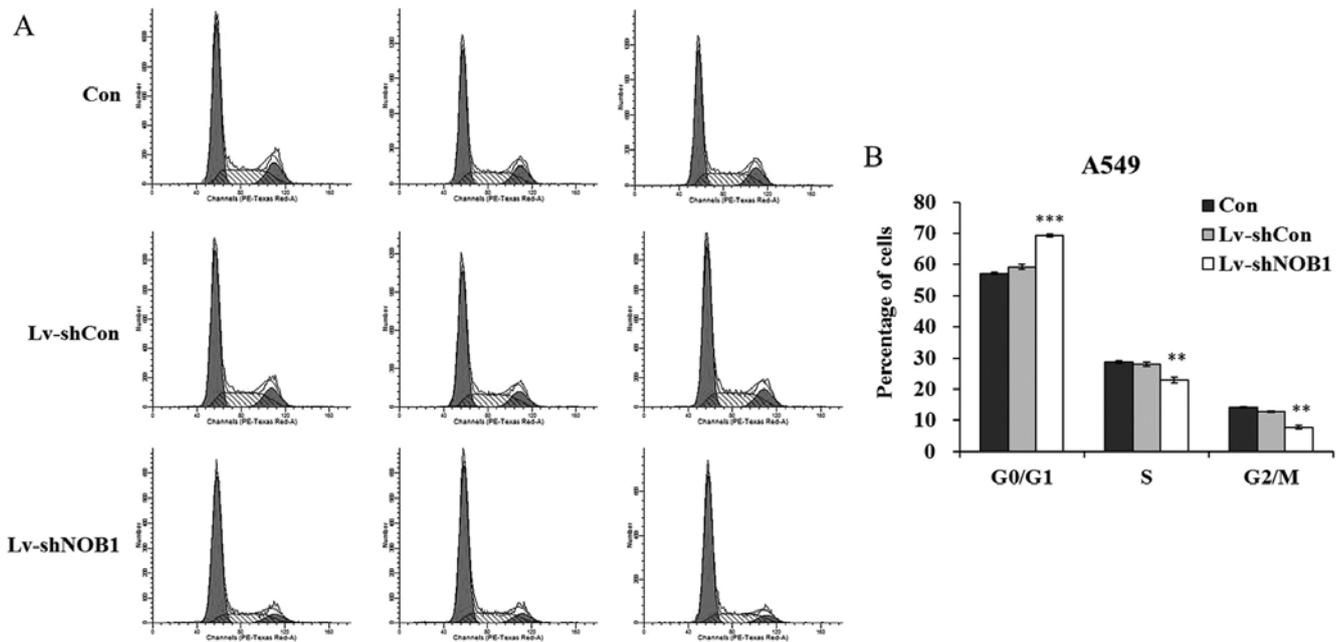


Figure 5. Effect of NOB1 knockdown on cell cycle progression of A549 cells. (A) Flow cytometric histograms of A549 cells under each cell cycle phase. (B) A549 cells were blocked in the G0/G1 phase in the Lv-shNOB1 group. **P<0.01, ***P<0.001 vs. the Lv-shCon group. Lv-shNOB1, NOB1 shRNA lentivirus; Lv-shCon, non-silencing shRNA lentivirus.

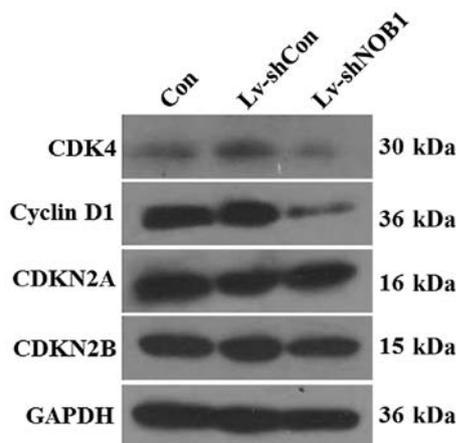


Figure 6. Western blot analysis of cyclin D1, CDK4, CDKN2A and CDKN2B expression in A549 cells. GAPDH was used as a control protein.

Discussion

NOB1 was first identified as an essential gene encoding NOB1p in *Saccharomyces cerevisiae*. NOB1p, as a nuclear protein in mammalian cells, has been proven to play a crucial part in proteasome biogenesis (8). In the present study, we found that NOB1 was highly expressed in the non-small cell lung cancer samples and ~13.8% of the non-small cell lung cancer samples exhibited aberrantly strong NOB1 expression. Recently, it was reported that *NOB1* is responsible for the high proliferation rate of cancer cells and repression of *NOB1* could suppress breast cancer and ovarian cancer cell survival (14,15). In the present study, *NOB1* was predicted to be an oncogenic factor in non-small cell lung cancer and silencing of *NOB1* may inhibit non-small cell lung cancer cell proliferation.

Non-small cell lung cancer has emerged as one of the leading causes of cancer-related death in the world. The influence of *NOB1* downregulation on the growth of two non-small cell lung cancer cell lines A549 and H1299 which express significantly high expression of *NOB1* was investigated. As a novel strategy in cancer treatment, gene-level approaches have generated increased attention. RNAi technology has been proven to be an efficient, specific and stable method to silence target genes (3). Taking advantage of the prevalence and availability of RNAi technology in cancer therapy as well as the relatively high and stable transfection rate of viral vectors (16), a lentivirus shRNA system was used to knock down *NOB1* expression in non-small cell lung cancer cells. The real-time PCR and western blotting results demonstrated that the expression of *NOB1* was sufficiently suppressed in the non-small cell lung cancer cells, which guaranteed the subsequent assays. The notably reduced proliferation of both non-small cell lung cancer cell lines was observed by MTT assay as the expression of *NOB1* was decreased. It was also confirmed that the colony formation capacity of the A549 cells was inhibited following knockdown of *NOB1*. *NOB1* silencing led to A549 cell cycle arrest in the G0/G1 phase, which contributed to the cell growth inhibition. Cyclin D1 and CDK4 are key molecules for G1-S and G2-M transition during the cell cycle, respectively (17,18). CDKN2A and CDKN2B are potent cyclin-dependent kinase inhibitors, and their induction may also cause cell cycle arrest (19). Western blotting showed that depletion of NOB1 resulted in a significant increase in CDKN2A expression, and a slight decrease in cyclin D1 and CDK4 expression, which contributed to cell cycle arrest. Therefore, *NOB1* plays an important role in the growth and cell cycle progression of non-small cell lung cancer cells.

The proteolysis of intracellular proteolysis is mainly through the ubiquitin-proteasome pathway, and the proteome is

confirmed to control various proteins involved in cycle progression and apoptosis such as the cyclins, caspases, nuclear factor κ B (NF- κ B) and apoptosis proteins (20,21). NOB1p facilitates the maturation of the 20S proteasome, and then regulates the biogenesis of the 26S proteasome which contributes to protein degradation by the ubiquitin-proteasome system (UPS) in universal biological processes including cell cycle progression in eukaryotes (22,23). Thus, the inhibitory effect on the proliferation of non-small cell lung cancer cells induced by *NOBI* repression may be attributed to the influences on degradation of cell cycle proteins and various complex aspects in cell cycle progression. Subsequent research is needed to elucidate the mechanism involved in the regulation of the cell cycle of non-small cell lung cancer cells by *NOBI* and its underlying relationship with proteasome-mediated degradation.

In conclusion, the present study demonstrated that lentivirus-mediated *NOBI* knockdown inhibited the growth of non-small cell lung cancer cells along with cell cycle arrest in the G0/G1 phase. These results suggest that *NOBI* may be considered as a potential target for non-small cell lung cancer therapy.

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