RESEARCH ARTICLE

Knockdown of HMGN5 Expression by RNA Interference Induces Cell Cycle Arrest in Human Lung Cancer Cells

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Abstract

HMGN5 is a typical member of the HMGN (high mobility group nucleosome-binding protein) family which may function as a nucleosomal binding and transcriptional activating protein. Overexpression of HMGN5 has been observed in several human tumors but its role in tumorigenesis has not been fully clarified. To investigate its significance for human lung cancer progression, we successfully constructed a shRNA expression lentiviral vector in which sense and antisense sequences targeting the human HMGN5 were linked with a 9-nucleotide loop. Inhibitory effects of siRNA on endogenous HMGN5 gene expression and protein synthesis were demonstrated via real-time RT-PCR and western blotting. We found HMGN5 silencing to significantly inhibit A549 and H1299 cell proliferation assessed by MTT, BrdU incorporation and colony formation assays. Furthermore, flow cytometry analysis showed that specific knockdown of HMGN5 slowed down the cell cycle at the G0/G1 phase and decreased the populations of A549 and H1299 cells at the S and G2/M phases. Taken together, these results suggest that HMGN5 is directly involved in regulation cell proliferation in A549 and H1299 cells by influencing signaling pathways involved in cell cycle progression. Thus, our finding suggests that targeting HMGN5 may be an effective strategy for human lung cancer treatment.

Keywords: HMGN5 - cell proliferation - cell cycle arrest - human lung cancer cells - RNA interference

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Introduction

Lung cancer is the leading cause of cancer death worldwide (Jemal et al., 2006). Despite surgical resection and advances in radiotherapy and chemotherapy, long-term survival rates remain extremely poor over the past years (Spiro and Silvestri, 2005). It has been established that lung cancer arises as a consequence of the accumulation of multiple genetic alterations involving critical genes that control cell proliferation and survival (Minna et al., 2002; Osada and Takahashi, 2002). Therefore, a better understanding of oncogenic signaling mechanism underlying lung cancer and the identification of new therapeutic targets for treatment of this disease are urgently needed.

High mobility group nucleosome-binding (HMGN) protein family contains five architectural non-histone chromosomal proteins, which specifically binds to the nucleosome core particle (CP) by their nucleosomal binding domain (NBD) in a sequence-independent manner and modulate the structure and function of chromatin (Bustin, 1999; Postnikov and Bustin, 2010; Zhu and Hansen, 2010). HMGNs have been studied extensively for their ability to modulate transcription (Furusawa et al., 2006; Zhu and Hansen, 2007; Ueda et al., 2009). HMGN5/NSBP1 is a typical member of the HMGN family which is localized to the nucleus and contains a functional NBD and a conserved motif in the C-terminus domain (Shirakawa et al., 2000). However, unlike the other HMGNs, HMGN5 contains a longer acidic C-terminal domain which affects cellular localization and architectural properties of the protein (Rochman et al., 2010). Alteration of the cellular levels of HMGN5 leads to widespread changes in the cellular transcription profile (Rochman et al., 2009; Rochman et al., 2011). In addition, HMGN5 overexpression had been observed in prostate cancer (Jiang et al., 2010), squamous cell carcinoma (Green et al., 2006), breast cancer (Li et al., 2006) and gliomas (Qu et al., 2011) in human, suggesting a role for HMGN5 in tumorigenesis (Gerlitz, 2010). Thus, HMGN5 might be a potential molecular target for cancer therapy. RNA interference (RNAi) is a method of gene silencing, which is induced by small interfering RNA (siRNA) or short hairpin RNA (shRNA). RNAi presents an innovative

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research approach for the analysis of gene function in mammalian cells (Brummelkamp et al., 2002). The purpose of the present study was to determine the effects of RNAi-mediated knockdown of HMGN5 expression on the cell proliferation and cell cycle progression in human lung cancer cell lines A549 and H1299, and to gain insight into the relevance of HMGN5 in lung cancer biology.

Materials and Methods

Reagents and antibodies

RPMI-1640 medium and calf serum were from Gibco BRL (Grand Island, NY); TRIzol Reagent and Lipofectamine 2000 were from Invitrogen (Carlsbad, CA); 5-Bromo-2'-deoxyuridine (BrdU) Cell Proliferation ELISA kit was from Roche Applied Sciences (Indianapolis, IN). Giemsa was from Chemicon International (Temecula, CA); Propidium Iodide (PI) was from Sigma-Aldrich (St. Louis, MO); bicinchoninic acid (BCA) Protein assay was from HyClone-Pierce (South Logan, USA); M-MLV Reverse Transcription was from Promega (Madison, WI); oligo-dT was from Sangon Biotech (Shanghai, China); SYBR green Master Mixture was from Takara (Otsu, Japan); pFH1UGW vector and virion-packaging elements were from Huangli Biol. (Shanghai, China). Anti-NSBP1 antibody (Cat# AV37788) was from Sigma-Aldrich. Mouse anti-GAPDH (Cat# sc-32233), Goat anti-Mouse IgG (Cat# sc-32233) and goat anti-rabbit IgG (Cat# sc-2030) were Santa Cruz biotechnology (Santa Cruz, CA). All other chemicals were of analytical grade.

Cell culture

Human lung cancer cell lines A549 (p53 wild-type) and H1299 (p53 null) were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown in a humidified incubator at 37 °C under 5% CO_2/95% air and used for assays during exponential phase of growth.

Construction of recombinant lentivirus

The recombinant lentivirus was constructed as previously described (Rubinson et al., 2003). In briefly, a 19-bp fragment within the HMGN5 cDNA was chosen as the target for HMGN5-siRNA: 5'-CACAGCCTTTCTTTAGCAT-3' (GenBank accession no. NM_030763.2). This sequence was submitted to a BLAST search against the human genome sequence to ensure that the human genome was not targeted. A nonsilencing fragment (5'-TTCTCCGAACGTGTCACGT-3') as negative control has no significant homology to mouse or human gene sequences. Short hairpin RNA (shRNA) fragments were hybridized with synthesized sense and antisense oligonucleotides. Then the hybridized shRNA fragments were cloned into the NheI/PacI restriction sites of the plasmid pFH1UGW which was driven by H1 promoter to yield plasmid pFH1UGW-HMGN5. Correct insertions of shRNA cassettes were confirmed by restriction mapping and direct DNA sequencing.

The siRNA containing plasmid (pFH1UGW-HMGN5), together with two imperative elements containing plasmids were co-transfected into 293T cells with lipofectamine 2000. The supernatant was collected and centrifuged (4,000 g, 10 min, 4 °C) 48 h later. After filtering the collected medium through 0.45 μM filters, the virus was concentrated by spinning at 4,000 g for 15 min following by a second spin (1,000 g, 2 min). The concentrated virus was stored at -80°C. The titer of lentivirus vectors was determined by dilution and using fluorescent microscopy (CKX41, Olympus).

Recombinant lentivirus Transfection in A549 and H1299 cells

A549 and H1299 cells were plated at 5×10^4 cell/well in 6-well plates. After 24 h of culture, recombinant lentivirus encoding siRNA against HMGN5 in serum-free growth medium was added at a multiplicity of infection (MOI) of 50. After 2 h at 37 °C and 5% CO_2, serum containing growth medium was added to the cells, and there was complete replacement of growth medium after 24 h. Then, 5 days post-transfection, reporter gene expression was examined using fluorescent microscopy (CKX41, Olympus).

Quantitative real-time RT-PCR analysis of HMGN5 mRNA expression

Total RNA was prepared using TRIzol reagent according to the manufacturer's instruction. The quantity and purity of the RNA were determined by detecting light absorbance at 260 nm (A_{260}) and the ratio of A_{260}/A_{280}. Then, 2 μg of total RNA was used to synthesize the first strand of cDNA using M-MLV Reverse Transcriptase. Real-time PCR reactions using SYBR Green Master Mixture were run on TAKARA TP800-Thermal Cycler Dice™ Real-Time System. β-actin was used as an internal control. The following primers were used: HMGN5: 5'-GGTTGTCTGCTATGCTTGTG-3' as forward and 5'-ACTGCTTCTTGCTTGGTTTC-3' as reverse; β-actin: 5'-GGCGGCACCACCATGTACCCT-3' as forward and 5'-AGGGGCCGGACTCGTCATACT-3' as reverse. Thermal cycling condition was subjected to 15 s at 95 °C and 45 cycles of 5s at 95 °C and 30s at 60 °C. Data was analyzed with TAKARA Thermal Dice Real Time System software Ver3.0. Relative quantification was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with normalization to β-actin.

Western blot analysis of HMGN5 protein expression

Cells were washed twice with ice-cold PBS and suspended in a lysis buffer (2% Mercaptoethanol, 20% Glycerol, 4% SDS in 100mM Tris-HCl buffer, pH 6.8). After incubated for 15 min on ice, cell extracts were prepared on ice by ultrasonic disruption. After centrifugation at 12,000 g for 15 min at 4 °C, the supernatants were collected, and the protein concentrations were determined using BCA Protein assay. Equal amounts of lysate were subjected to SDS-PAGE. After electrophoresis, protein blots were transferred onto PVDF membrane using an electro-blotting apparatus (Tanon, Shanghai, China). The membrane was blocked with 5% nonfat milk in TBST

solution, and incubated overnight with the corresponding primary antibodies in the blocking solution at 4 °C. After 3 washes with TBST solution, the membrane was incubated at room temperature for 2 h, with horseradish peroxidase-conjugated secondary antibody diluted with TBST solution (1:5000). The signals of detected proteins were visualized on ECL plus Western blotting detection system (Amersham). GAPDH protein levels were used as a control to verify equal protein loading.

MTT assay

Cell proliferation was evaluated by MTT assay. The test cells in exponential growth were plated at a final concentration of 2 × 10^3 cells/well in 96-well culture plates for different culture time. MTT (10 μL, 10 mg/ml) was then added. After an additional 4 hr of incubation, the reaction was terminated by removal of the supernatant and addition of 150 μl DMSO for 30 min. Optical density (OD) of each well was measured at 490 nm using ELISA reader (ELx808 Bio-Tek Instruments, USA).

Determination of cell proliferation by BrdU incorporation assay

Cell proliferation was determined using a cell proliferation ELISA, BrdU (colorimetric) kit, following the instructions of the manufacturer. In brief, the transfected cells were seeded in 96-well plates at a density of 2 × 10^3 cells/well. Ten microliters of BrdU labeling solution were added to each well, and cells were incubated at 37 °C. After an incubation period of 2 h, the culture medium was removed and the cells were treated with 200 μl of Fixdenat solution at room temperature for 30 min in the dark. The Fixdenat solution was removed and the cells were treated with 5-10% BSA to block non-specific binding 30 min at room temperature in the dark. Then, the BSA solution was removed and the cells were incubated with 100 μl of anti-BrdU-POD labeling solution at room temperature for 90 min in the dark. The anti-BrdU-POD solution was removed and the cells were washed three times the BrdU washing solution. The substrate solution (100 μl) was added to the cells at room temperature for 30 min in the dark and then 10 μl 10% H2SO4 were added to each well. The color developed was measured in a microplate reader at a wavelength of 490 nm. Growth rate was quantified by the following equation: Growth rate (%) = $(A_{72\,h} - A_{24\,h})/A_{24\,h} \times 100$, where $A_{72\,h}$ and $A_{24\,h}$ referred to the BrdU incoporation level (A490 nm) after 72 h and 24 h of culture, respectively.

Colony formation assay

Cell growth and survival ability was also determined by the plate-colony-formation assay. In brief, 200 transfected cells were plated in 6-well plates. Cells were cultured for 14 days at 37 °C under 5% CO_2. Culture medium was changed at 3-day intervals. Afterward, cells were fixed with 4% paraformaldehyde for 30 min at room temperature. The colonies were stained with Giemsa for 15 min, then washed with water and air-dried. The number of cell colonies was counted using a light microscopy.

Cell cycle analysis

Cell cycle distribution was assessed by staining DNA content with PI as previously described method (Krishan, 1975). Briefly, the transfected cells were harvested by trypsinization, centrifuged at 1000 rpm for 5 min, washed with PBS and fixed in 70% ethanol at 4 °C for at least 1 h. Cells were collected by centrifugation and resuspended in PBS containing 100 μg/ml RNase A and 40 μg/ml PI, and then incubated at 4 °C for 30 min, protected from light. Cells were analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA). The fractions of the cells in G0/G1, S, and G2/M phases were analyzed using dedicated software (Becton-Dickinson, San Jose, CA).

Statistical analysis

The values are expressed as the mean of at least three different experiments ± S.D. The results were analyzed by Student's t-test, and P<0.05 was considered statistically significant.

Results

Lentivirus-mediated RNAi inhibited HMGN5 mRNA and protein expression in A549 and H1299 cells

To investigate whether HMGN5 plays an important role in human lung cancer cell proliferation and survival,

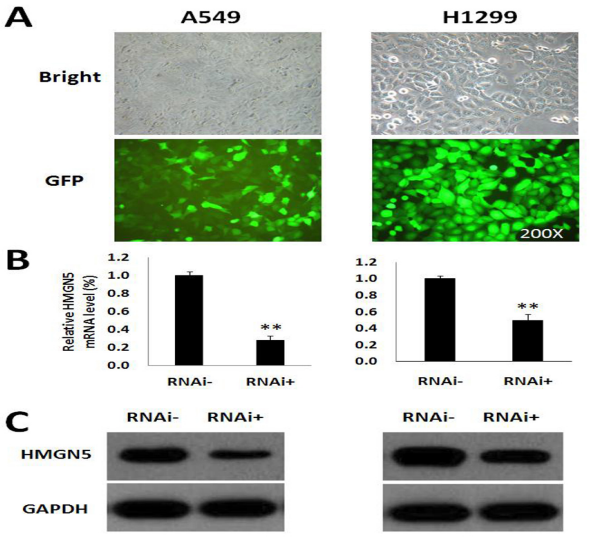

Figure 1. Lentivirus-mediated RNAi Stably Decreased HMGN5 Expression in A549 and H1299 Cells. (A) Transduction efficiency was estimated 5 days after infection at MOI of 50 in A549 cells, and transduction efficiency was estimated 5 days after infection at MOI of 15 in H1299 cells. Light micrograph (top); Fluorescent micrograph (bottom) (×200). High intensity of the fluorescence image indicated high efficiency of the transfection. (B) Total RNA was extracted at 5 days after infection and Relative HMGN5 mRNA expression was determined by quantitative real-time RT-PCR.β-actin was used to an internal gene. Data represent the mean ± S.D. of three independent experiments. *P<0.05, **P<0.01, compared with RNAi- group; (C) Total cellular proteins were extracted at 5 days after infection and determined by western blot analysis using antibodies against HMGN5 and GAPDH as an internal control. Data represent one out of three separate experiments. RNAi-: cells transfected with lentivirus-medtiaed Con-siRNA; RNAi+: cells transfected with lentivirus-mediated HMGN5-siRNA

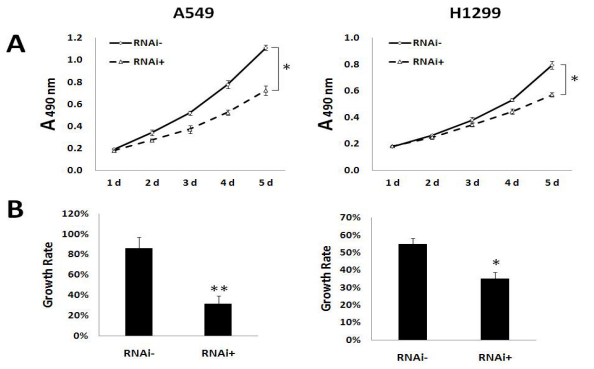

Figure 2. Effect of HMGN5 Silencing on A549 and H1299 Cell Proliferation. (A) Cell viability was measured by MTT assay once daily for 5 days. Cell proliferation was expressed as the absorbance values. (B) Growth rate was expressed as the % BrdU incorporation at 5 days after transfection. *p<0.05, **p<0.01 compared to RNAi- group. RNAi-: cells transfected with lentivirus-mediated Con-siRNA; RNAi+: cells transfected with lentivirus-mediated HMGN5-siRNA

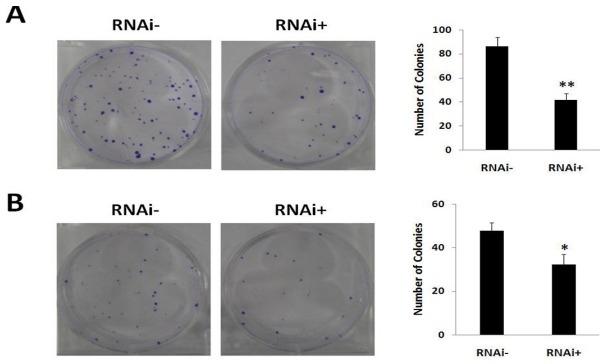

Figure 3. Detection of Cell Proliferation by Plate Colony Formation Assay in A549 and H1299 Cells. (A) A549 cells were seeded at 200 per well and allowed to form colonies. (B) H1299 cells were seeded at 200 per well and allowed to form colonies. Data represent the mean ± S.D. of three independent experiments. *P<0.05, **P<0.01, compared to RNAi- group. RNAi-: cells transfected with lentivirus-mediated Con-siRNA; RNAi+: cells transfected with lentivirus-mediated HMGN5-siRNA

A549 and H1299 cells were transfected with lentivirus-mediated siRNA targeting HMGN5. Transfection efficiency of A549 and H1299 cells was determined by detecting GFP expression using fluorescence microscopy. More than 90% of A549 cells were transfected at 5 days post-transfection at MOI of 10, and more than 90% of H1299 cells were transfected at 5 days post-transfection at MOI of 15. They were indicated by the expression of GFP (Figure 1A)

The mRNA and protein expression of HMGN5, inhibited by lentivirus-mediated siRNA in A549 and H1299 cells, were analyzed by real-time RT-PCR and western blotting. After 5 days of infection, cells were collected and lysed for analysis. As shown in Fig. 1B, the level of HMGN5 mRNA in A549 and H1299 cells transfected with lentivirus-mediated HMGN5-siRNA ($RNAi^+$) was significantly decreased by 70% and 50% compared with those transfected with lentivirus-mediated con-siRNA (RNAi-), respectively (**P<0.01). Additionally, western blotting also showed that the HMGN5 protein expression levels in A549 and H1299

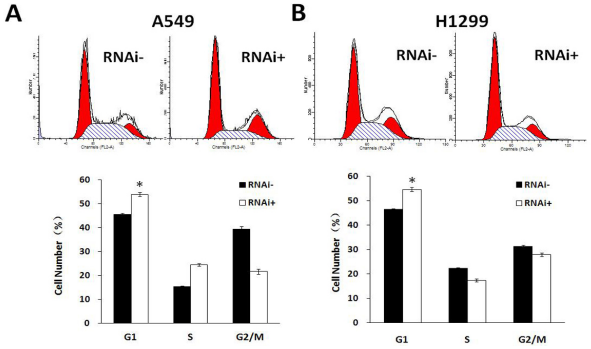

Figure 4. Effect of Downregulation of HMGN5 Expression on Cell Cycle Progression in A549 and H1299 Cells. Cell cycle distribution was performed by flow cytometric analysis. (A) Knockdown of HMGN5 by RNAi in A549 cells induced cell cycle arrest in G0/G1 phase at 5 days after transfection. (B) Knockdown of HMGN5 by RNAi in H1299 cells induced cell cycle arrest in G0/G1 phase at 5 days after transfection. Data represent the mean ± S.D. of three independent experiments. *P<0.05, **P<0.01, compared with RNAi- group. RNAi-: cells transfected with lentivirus-mediated Con-siRNA; RNAi+: cells transfected with lentivirus-mediated HMGN5-siRNA

cells were significantly reduced in RNAi+ group, compared with RNAi- group (Figure 1C). The above results indicated that the expression of HMGN5 could be downregulated specifically and effectively by lentivirus-mediated siRNA against HMGN5 in A549 and H1299 cells.

Effect of HMGN5 downregulation on A549 and H1299 cell proliferation

To further evaluate whether silencing HMGN5 gene in A549 and H1299 cells may inhibit cell growth and proliferation, MTT, BrdU incorporation and colony formation assay were performed. Downregulation of HMGN5 expression decreased viability of A549 and H1299 cells in a time-dependent manner. After 5 days of infection, the percentages of viable cells in A549 and H1299 cells transfected with lentivirus-mediated HMGN5-siRNA (RNAi+) respectively decreased 30 % and 25%, as compared to the cells transfected with lentivirus-mediated Con-siRNA (RNAi-) (Figure 2A, **P<0.01 and *P<0.05, respectively). Furthermore, the rate of DNA synthesis in the A549 and H1299 cells infected with HMGN5-specific RNAi was significantly suppressed, compared to control cells (Figure 2B, **P<0.01 and *P<0.05, respectively).

In addition, the results of colony formation assay also showed that lentivirus-mediated siRNA against HMGN5 caused a significant decreased in the number of colonies in A549 and H1299 cells, as compared to RNAi- group (**P<0.01 and *P<0.05, respectively, Figure 3A and 3B). All these results indicated that downregulation of HMGN5 expression by RNAi significantly inhibited cell growth and proliferation in A549 and H1299 cells.

Effect of HMGN5 downregulation on cell cycle progression in A549 and H1299 cells

To explore the possible underlying mechanisms of HMGN5 downregulation in inhibiting A549 and H1299 cell proliferation, we used flow cytometry to examine

if the cell cycle was affected. In comparison with cells transfected with lentivirus-mediated Con-siRNA (RNAi-), there were great changes in cells transfected with lentivirus-mediated HMGN5-siRNA (RNAi+). As shown in Figure 4A,, the knockdown of HMGN5 induced an increase in the percentage of cells in the G0/G1 phase of the cell cycle, parallel to a decrease in the percentage of cells in the S-phase. Similar results were also seen in H1299 cells (Figure 4B). These results indicate that HMGN5 knockdown can inhibit A549 and H1299 cell proliferation by the induction of G0/G1 arrest.

Discussion

The dynamic architecture of the chromatin fiber plays a key role in regulating transcriptional process necessary for proper cell function and integration of endogenous and exogenous signals for proper adjustment of cellular response to changing environment. HMGN5, a chromatin architectural protein, belongs to the HMGN protein family, which is a key regulator of chromatin structure and transcription (Bustin, 2001; Rochman et al., 2010). Thus, its aberrant expression is deleterious for the cell fate. Recently, there are some reports to show that HMGN5 is overexpressed in several human tumor cell lines, knockdown of which induced cell cycle arrest and apoptosis (Green et al., 2006; Li et al., 2006; Jiang et al., 2010; Qu et al., 2011). Therefore, HMGN5 may play an important role in cancer progression.

Current lentiviral technology has facilitated a wide range of loss- and gain-of- function genetic studies in mammalian systems (Meerbrey et al., 2011). In the present study, we employed the lentivirus-mediated siRNA to knockdown HMGN5 expression and investigated the effects of HMGN5 silencing on the cell proliferation and cell cycle progression in human lung cancer cell lines A549 and H1299. More than 90% of A549 cells were transfected at 5 days post-transfection at MOI of 10, and more than 90% of H1299 cells were transfected at 5 days post-transfection at MOI of 15. They were indicated by the expression of GFP (Figure 1A). Real-time PCR and western blotting analysis indicated that the expression of HMGN5 gene could be downregulated specifically and effectively by siRNA-targeted HMGN5 in A549 and H1299 cells (Figure 1B and 1C). As shown in Figure 2 and Figure 3, we found that silencing of HMGN5 inhibited cell proliferation and reduced colony formation of A549 and H1299 cells. We also observed cells were arrested in the G0/G1 phase of cell cycle in A549 and H1299 cells transfected with lentivirus-mediated siRNA against HMGN5 by flow cytometry (Figure 4). These results implied that HMGN5 may play an important role in cell growth control and cell cycle progression of human lung cancer cells.

P53 is a cellular gatekeeper for the cell growth and division (Bates and Vousden, 1996; Levine, 1997; Hofseth et al., 2004). It has been shown that p53 can regulate cell cycle arrest, apoptosis, and DNA repair in a variety of cells (Levine, 1997; Hofseth et al., 2004). To confirm the role of p53 in the HMGN5 silencing inhibition of cell proliferation, human lung cancer cell lines A549 with p53 null and H1299 with p53 wild-type were selected as the targets for this study. The data indicated that HMGN5 silencing inducing cell cycle arrest in G0/G1 phase in A549 and H1299 cells may result from a p53-independent pathway. In addition, HMGN5 binds to nucleosomes via NBD, unfolds chromatin and affects transcription (Rochman et al., 2010). Thus, we presumed that HMGN5 may be targeted to specifically alter the expression of cell cycle-related gene in lung tumorigenesis. However, further experimental studies are needed to clarify the mechanism for HMGN5 silencing inducing cell cycle arrest in A549 and H1299 cells.

In conclusion, we successfully used a lentiviral system to induce a long-last down-regulation of HMGN5 in human lung cancer cell lines A549 and H1299. Knockdown of HMGN5 significantly inhibited A549 and H1299 cell proliferation by inducing cell cycle arrest in G0/G1 phase. Thus, HMGN5 is expected to be a potential therapeutic target for the treatment of lung cancer.

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