Am J Cancer Res 2018;8(8):1427-1439
www.ajcr.us /ISSN:2156-6976/ajcr0082928

*Original Article*

# The lncKLF6/KLF6 feedback loop regulates the growth of non-small cell lung cancer

Fei Li, Qianyun Zhang, Yange Gong, Jinxiang Yu

*Department of Pulmonary and Critical Care Medicine (PCCM) ward II, Hebei Cangzhou Central Hospital, Hebei Province, China*

Received July 15, 2018; Accepted July 30, 2018; Epub August 1, 2018; Published August 15, 2018

**Abstract:** Non-small lung cancer (NSCLC) is one of the most common causes of cancer-associated death worldwide. Long noncoding RNAs (lncRNAs) regulate cancer initiation and progression through different mechanisms. In the present study, we characterized a novel lncRNA named lncKLF6, which was upregulated in NSCLC and associated with poor clinical outcomes. lncKLF6 inhibited Kruppel-like factor 6 (KLF6) transcription and then facilitated NSCLC growth. lncKLF6 is associated with the epigenetic repressor BMI1 and regulates its stability via recruiting deubiquitinase USP22. Moreover, it was revealed that lncKLF6 was a KLF6-responsive lncRNA, as KLF6 could occupy the lncKLF6 promoter to facilitate its transcription. The negative feedback loop of lncKLF6 and KLF6 continuously enhanced the oncogenic effects. Thus, our study elucidates the mechanism of lncKLF6-mediated growth via suppression of KLF6, which provides the promising target for developing new therapeutic strategy in NSCLC.

**Keywords:** KLF6, deubiquitin, USP22, growth, feedback

## Introduction

Lung cancer is one of the most common causes of cancer-associated death worldwide [1, 2]. Small cell lung cancer (SCLC) and non-small lung cancer (NSCLC) account for 15% and 85% of lung cancer cases, respectively. Though the advancement of diagnostic and therapeutic technologies has been achieved, the 5-year overall survival rate of NSCLC remains as low as 40% [3]. Therefore, it is of great necessity to reveal the underlying mechanism of the initiation and progression of NSCLC for obtaining novel potential therapeutic targets.

With the development of high-resolution sequencing technology, thousands of long noncoding RNAs (lncRNAs) were recently identified. lncRNAs have been reported to be closely associated with various cellular processes, including cell proliferation, migration, invasion, autophagy and pluripotency [4, 5]. lncRNAs exert their functions through different mechanisms. For example, lncRNA HOTAIR was associated with E3 ligase Mex3b to regulate the ubiquitination of Runx3 and then enhance the invasion of gastric cancer [6]. HOTAIR upregulated Bcl2 expression and promoted the cellular proliferation through acting as a sponge for miR-143-3p in cervical cancer [7]. HOTAIR also interacted with histone-lysine N-methyltransferase enzyme EZH2 to epigenetically silence miR-34a expression and then induce epithelial-to-mesenchymal transition [8]. Recently, several lncRNAs were reported as potential prognostic and prognostic markers for NSCLC [9, 10]. However, the comprehensive pathophysiological contributions of lncRNAs to NSCLC and the underlying mechanisms remain unclear.

Ubiquitination is involved in both protein substrates and organelles. Ubiquitination of protein substrates is a reversible process. Deubiquitinases (DUBs) mediate removal of ubiquitin monomers or polymers from target proteins. Many DUBs, such as Ubiquitin specific peptidase 9 X linked (USP9X), USP10 and USP14, demonstrate the specificity for proteins involved in disease status. It has been shown that USP22 is overexpressed in several cancers [11, 12]. USP22 deubiquitinates protein substrates which are essential in tumor cell signaling and survival, and then protect them from proteasomal degradation. However, to date, the relationship between lncRNAs and DUBs is still elusive. In the present study, a novel lncRNA

named lncKLF6 is described, and it promotes NSCLC growth through suppression of Kruppel like factor 6 (KLF6) transcription via association with BMI1. Furthermore, it was found that lncKLF6 recruits DUB USP22 to enhance BMI1 stability and then suppresses KLF6 expression. Moreover, KLF6 suppresses lncKLF6 transcription in turn to sustain the oncogenic effects continuously.

## Materials and methods

### *Tissue samples and cell culture*

70 pairs of NSCLC and corresponding nontumoral lung tissues were obtained from patients who underwent surgery at Cangzhou Central Hospital. All the samples were immediately frozen in liquid nitrogen and stored at -80°C. Our study was approved by the Research Ethics Committee of Cangzhou Central Hospital and in accordance with the Declaration of Helsinki and written informed consent was obtained from all patients.

Two human NSCLC cell lines, A549 and 95D, were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium (Gibco) with 10% fetal bovine serum (FBS) (Gibco) at 37°C in a 5% $CO_2$ atmosphere.

### *lncKLF6 knockdown and overexpression*

pLKO.1 plasmid expressing lncKLF6 or KLF6 shRNAs were constructed by Genchem Biotechnology company (Shanghai, China). The target sequences of shRNAs were shown as follow: shlncKLF6-1: CACAAATAGCTTGGCCCTA; shlncKLF6-2: CAGGAATCCTAGAGGTAGT; shKLF6: CTGCTATGTTTCAGCCTCA. Scramble shRNA was used as negative control (shCon). The full-length lncKLF6 were cloned into pLV plasmid. Lentiviral vectors and packaging vectors were transfected into the packaging cell line 293T (ATCC) using the Lipo3000 Transfection Reagent (Invitrogen). The medium containing lentivirus was harvested. Then the NSCLC cells were infected with lentivirus. The stable cells were selected by puromycin.

### *siRNA transfection*

siRNA oligonucleotides were ordered from Ribobio Company (Guangzhou, China). Cells were transfected with siRNAs by using the Lipofectamine RNAiMAX transfection reagent (Invitrogen). The target sequences of siRNAs were shown as follow: siKLF6: CTGCTATGTTTCAGCCTCA; siUSP22: GTGCCAGGACTACATCTAT; siBMI1: AGCTAAATCCCCACCTGAT.

### *5' and 3' RACE*

ExactSTART RACE kit (Epicentre Biotechnologies) was used for RACE analysis. 5' and 3' RACE were performed according to the manufacturer's instruction.

### *Isolation of cytoplasmic and nuclear RNA*

Cytoplasmic and nuclear RNA were isolated and purified using the Cytoplasmic & Nuclear RNA Purification Kit (Norgen, Belmont, CA) according to the manufacturer's instructions.

### *Quantitative real-time (qRT-PCR)*

Total RNA was isolated using TRIzol Reagent (Invitrogen) and reverse-transcribed using a High Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturer's instructions. qRT-PCR analysis was performed using SYBR Green qPCR SuperMix kits (Life Technologies) on Roche Lightcycler 96 instrument. The primer sequences were provided as follow: KLF6-F: TTAACGGCTGCAGGAAAGT, KLF6-R: TTCCCATGAGCATCTGTAAGG; lncKLF6-F: CTCATGGGTACATTCAGAGAAGAG; lncKLF6-R: GAAAGACCACAGCGTCTACTAC.

### *RNA immunoprecipitation (RIP)*

RIP assays were performed by EZ-Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer's instructions. Briefly, cells were lysed in lysis buffer containing a protease inhibitor cocktail and RNase inhibitor. Magnetic beads were pre-incubated with negative control IgG, anti-BMI1 or anti-USP22 antibody for 1 hour at room temperature, and lysates were immunoprecipitated with beads at 4°C overnight. RNA was purified from RNA-protein complexes bound to the beads and was then analyzed by qRT-PCR.

### *Chromatin immunoprecipitation (ChIP)*

ChIP assays were performed by EZ-ChIP-Chromatin Immunoprecipitation (Millipore) according to the manufacturer's instructions. Briefly, crosslinked chromatin was sonicated into 200- to 1,000-bp fragments. The chromatin was immunoprecipitated using anti-BMI1 (Abcam),

anti-ubH2AK119 (Millipore), or anti-KLF6 antibodies (Abcam). DNA was purified from DNA-protein complexes bound to the beads and was then analyzed by qRT-PCR.

*RNA pull-down assay*

RNA pull-down assay was performed as previously described [13]. Briefly, RNAs were biotin-labeled with the Biotin RNA Labeling Mix (Roche) and in vitro transcribed using T7 RNA polymerase (Roche), which was treated with RNase-free DNase I (Roche) and purified utilizing the RNeasy® Plus Mini Kit (Qiagen). Biotinylated RNAs were mixed and incubated with NSCLC cell lysates. Streptavidin agarose beads (Life Technologies) were added to each binding reaction, followed by a 1-hr incubation period at room temperature. The beads were then boiled in sodium dodecyl sulfate (SDS) buffer. The eluted proteins were detected by western blot analysis.

*Co-immunoprecipitation*

Cells were lysed with an IP lysis buffer (Beyotime Institute of Biotechnology). Total protein was incubated with Dynabeads Protein G and the primary antibodies on a rocking platform overnight at 4°C. The immunoprecipitates were washed three times with the lysis buffer. After the loading buffer was added, the beads were boiled and subjected to western blot analysis.

*Deubiquitination assay*

Endogenous BMI1 was immunoprecipited using the anti-BMI1 antibodies (Millipore), respectively, in denaturing conditions. The BMI1 protein was purified and immunoblotted with anti-ubiquitin (Santa Cruz) antibodies.

*Cell proliferation analysis*

Cells with different treatments were seeded at 2000 cells per well in 96-well culture plates. At the indicated time points, 10 μl CCK-8 (Dojindo) was added to each well and incubated for 1.5 h. Then, the absorbance values (OD 450 nm) were measured using a spectrophotometer (Thermo Fisher Scientific).

*Tumorigenicity assay in vivo*

Briefly, 4-week-old male athymic nude mice were blinded and randomly divided into different groups, and each group had 6 mice (n = 6). The mice were subcutaneously injected with 1 × $10^7$ A549 cells with silencing of lncKLF6 to establish xenograft tumors. Tumor volume and mice weight were measured at the indicated times. All animal experiments were approved by the Institutional Animal Care and Use Committee at Cangzhou Hospital.

*Colony formation assays*

Cells were seeded at 2,000 cells per well in the six-well culture plates. After 10 days' culture, cells were fixed and stained with crystal violet.

*Cell cycle analysis*

Cells were collected and resuspended in 75% ethanol at 4°C overnight. The cells were collected by centrifugation and washed with PBS. Finally, the cells were resuspended in PBS containing 100 g/ml RNase A and 50 g/ml propidium iodide (PI). After incubation for 30 min at 37°C, samples were subjected to flow cytometry for cell cycle analysis, and the data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

*Cell apoptosis analysis*

Cells were trypsinized and resuspended, and then stained with fluorescein isothiocyanate-conjugated Annexin V and 7-AAD (Apoptosis Detection Kit, KeyGEN, Nanjing, China) according to the manufacturer. Cells were subjected to flow cytometry, and the data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

*Protein isolation and western blot*

Cells were lysed in RIPA buffer (Beyotime Institute of Biotechnology). Total proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore). Membranes were blocked in 5% non-fat milk and then incubated with anti-BMI1 (Cell Signaling), anti-GAPDH (Proteintech), anti-KLF6 (Cell Signaling), anti-USP22 (Cell Signaling) or anti-Ubiquitin (Cell Signaling) antibodies, followed by incubation with secondary antibodies conjugated with horseradish peroxidase (HRP, Jackson). Immunoreactive proteins were visualized using the Western Chemiluminescent HRP Substrate (ECL) (Millipore).

*Luciferase assays*

The lncKLF6 promoter were constructed into pGL3-based plasmids. Cells were transfected

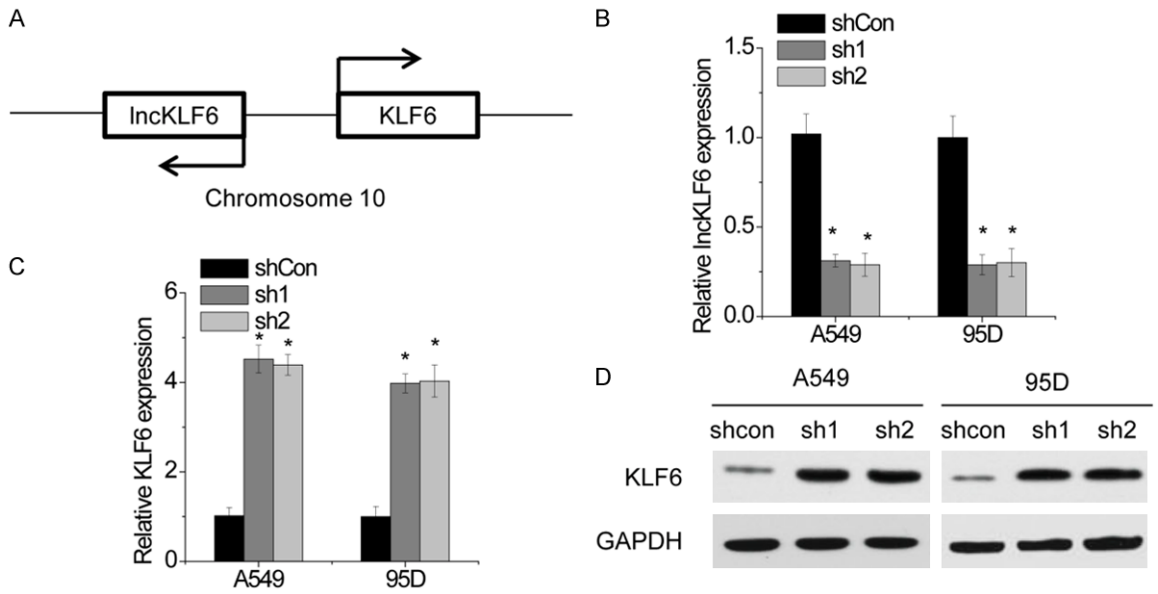


**Figure 1.** lncKLF6 suppresses KLF6 transcription. A. The location of *lncKLF6* and *KLF6* gene. The arrow indicated the transcriptional orientation. B. A549 and 95D cells were transfected with lentivirus expressing control shRNA (shcon) or lncKLF6 shRNAs (sh1 and sh2). The knockdown efficiency was examined by qRT-PCR. C. The KLF6 mRNA expression was determined in control and lncKLF6 knockdown NSCLC cells by qRT-PCR. D. The KLF6 protein expression was determined in control and lncKLF6 knockdown NSCLC cells by western blot. *P<0.05.

with pGL3-based reporter constructs as described, and pRLSV40 was the internal control plasmid. After transfection 36 h, the cells were lysed and assayed via a dual-luciferase reporter assay kit (Promega, Madison, WI, USA). Luciferase activity was normalized to that of the co-transfected pRL-SV40 plasmid.

*Statistical analysis*

The differences in the results between groups were compared using t-test or ANOVA test. Analysis was performed using the SPSS (version 18.0), and *P* values less than 0.05 were considered significant.

## Results

*lncKLF6 suppresses KLF6 transcription*

Firstly, the microarray data was analyzed from Gene Expression Omnibus data sets (GSE19-188 and GSE18842). In previous study, it was demonstrated that intergenic lncRNAs could regulate the transcription of neighboring genes *in cis* or *in trans* [14]. Therefore, we screened the intergenic lncRNAs which were overexpressed in NSCLC tissue and located in the nearby coding genes associated with NSCLC progression. KLF6 is frequently downregulated, functions as a tumor suppressor to inhibit cell proliferation and invasion and induces apoptosis in several types of cancers, including NSCLC [15, 16]. It was also found that the uncharacterized intergenic lncRNA was termed as lncKLF6 (gene symbol: RP11-482E14 or AL357833), which resided on chromosome 10, located in the upstream of the *Kruppel-like factor 6* (*KLF6*) gene and was upregulated in NSCLC tissue (**Figure 1A**). We knockdown the lncKLF6 expression in two NSCLC cell lines, A549 and 95D cells, and the knockdown effect was detected by qRT-PCR (**Figure 1B**). Notably, lncKLF6 knockdown significantly increased both mRNA and protein levels of its nearby protein-coding gene *KLF6* (**Figure 1C** and **1D**). Together, novel lncRNA lncKLF6 that suppresses KLF6 transcription was identified.

*lncKLF6 interacts with BMI1 protein and enhances its stability*

Next, we explored the molecular mechanisms by which lncKLF6 inhibited KLF6 transcription. A total length of 2308 nt of lncKLF6 transcription was determined by a RACE assay (**Figure 2A**). Cellular fractionation assays also showed that lncKLF6 was mainly localized in the nuclei of NSCLC cells (**Figure 2B**). The nucleic lncRNA

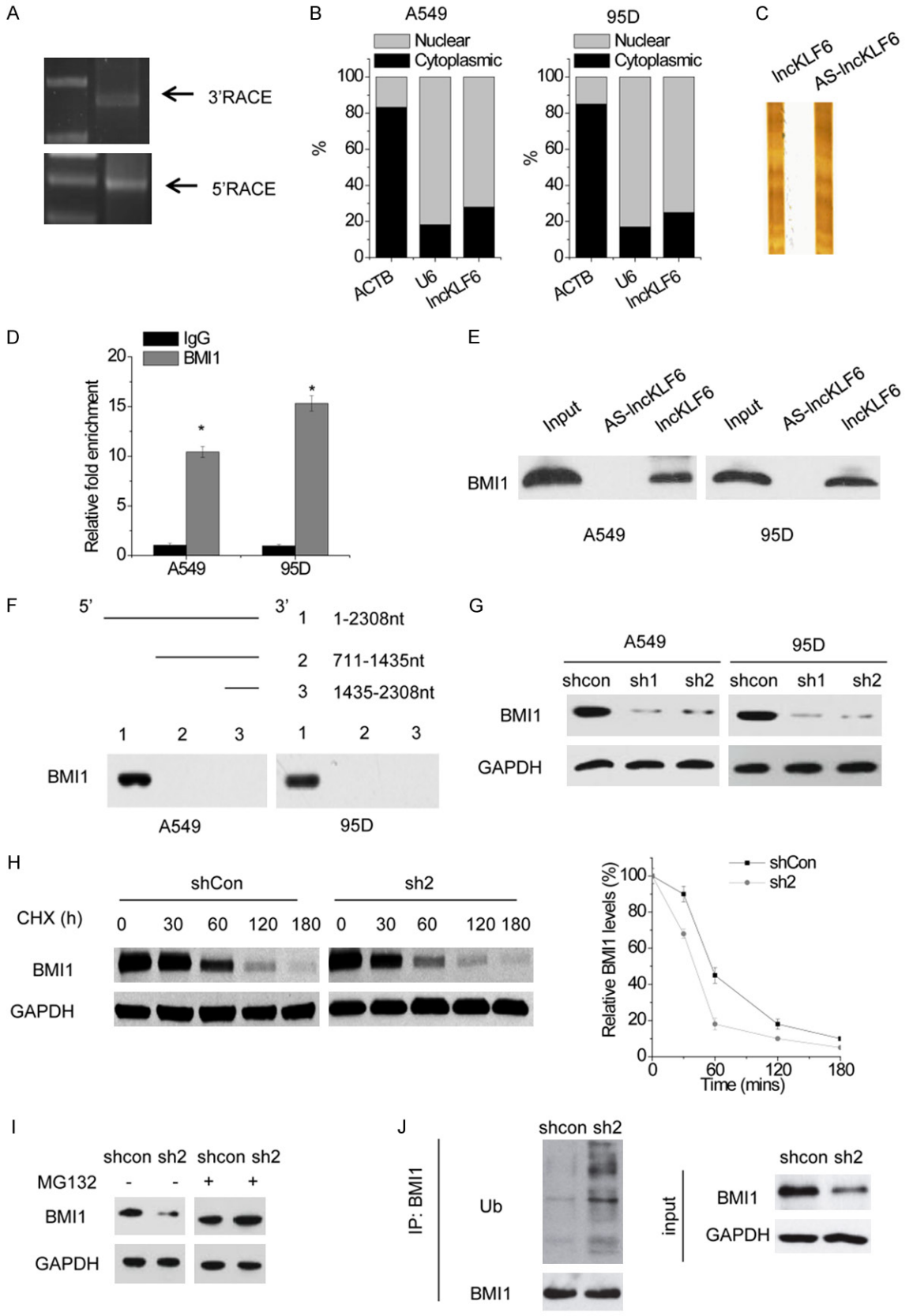


**Figure 2.** lncKLF6 interacts with BMI1 protein and enchances its stability. A. Representative image of PCR products from the 5'-RACE and 3'-RACE procedure. The major PCR product is marked by an arrow. B. The cellular location of

lncKLF6 in NSCLC cells. C. Biotin-RNA pull-downs were performed with nuclear extracts of A549 cells using sense lncKLF6 transcript and antisense lncKLF6 (AS-lncKLF6). This was followed by mass spectrometry. D. The association between lncKLF6 and BMI1 was determined by RIP and followed by qRT-PCR. IgG was taken as negative control. E. The association of lncKLF6 and BMI1 were confirmed through RNA pull-down assay. Antisense lncKLF6 was taken as negative control. F. Western blot of BMI1 in protein samples pulled down by different lncKLF6 fragments. G. The effect of lncKLF6 knockdown on BMI1 protein level. H. A549 cells with lncKLF6 were treated with 30 mg/mL of cycloheximide (CHX), and harvested at the time points indicated after CHX treated. Immunoblot for BMI1 is shown. I. BMI1 protein levels in lncKLF6-silencing A549 cells treated with vehicle control or MG132. J. BMI1 ubiquitination level in lncKLF6-silencing A549 cells treated with MG132. *P<0.05.

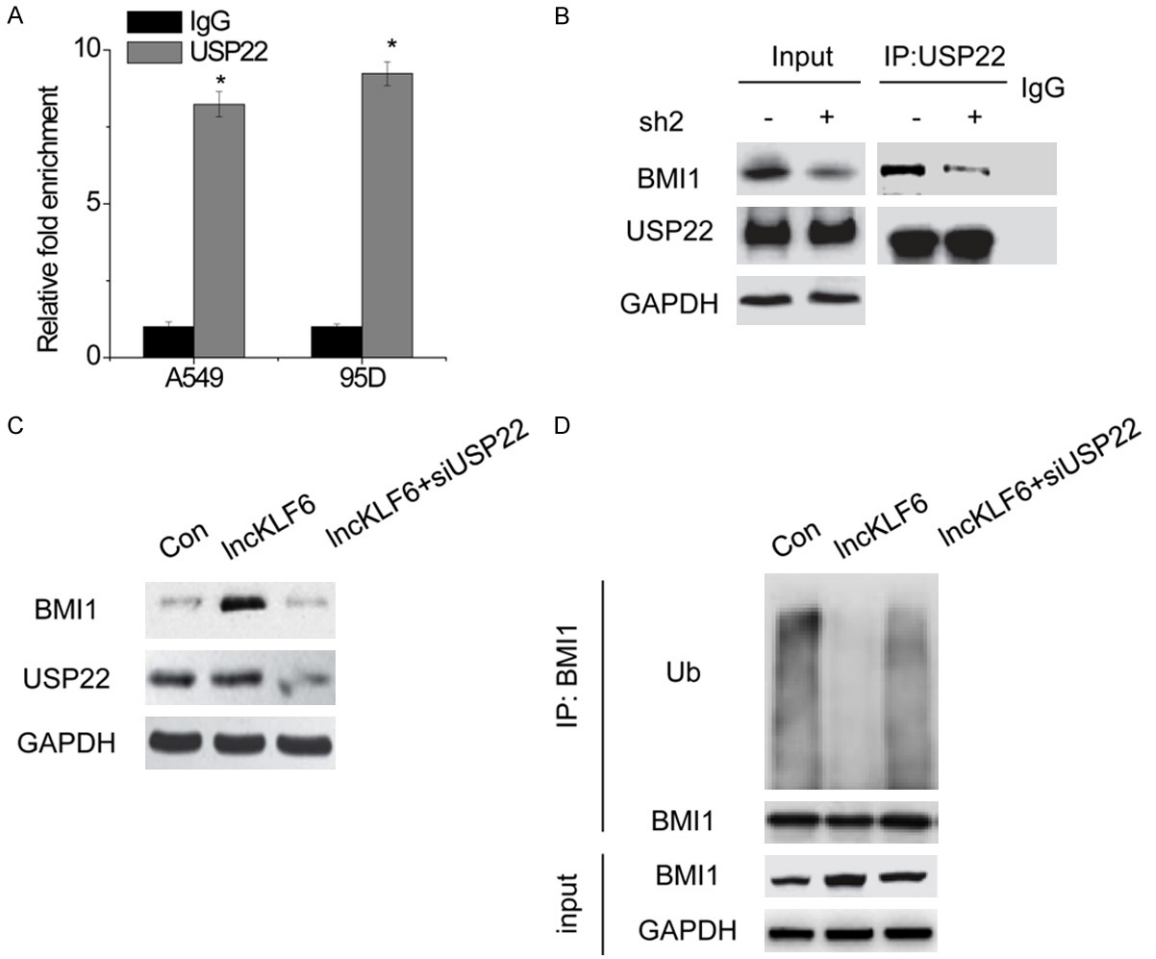


**Figure 3.** lncKLF6 stabilize BMI1 through recruiting deubiquitinase USP22. A. The RIP assay was performed to examine the interaction of USP22 and lncKLF6. B. The interaction between USP22 and BMI1 was decreased by lncKLF6 knockdown. C. USP22 knockdown abolished the BMI1 upregulation mediated by lncKLF6 overexpression. D. USP22 knockdown rescued the BMI1 ubiquitination level inhibited by lncKLF6 overexpression. *P<0.05.

usually regulated gene expression through interaction with RNA binding proteins [17]. Therefore, a RNA pull-down assay followed by mass spectrometry analysis is performed to identify proteins that interacted with lncKLF6 (**Figure 2C**). BMI1, a core component of polycomb repressive complex 1 (PRC1), was recognized. To validate the interaction between lncKLF6 and BMI1, the RIP assay was performed by using an anti-BMI1 antibody. The results showed that lncKLF6 was significantly enriched by the BMI1 antibody compared with the negative control IgG (**Figure 2D**). To further confirm the association between lncKLF6 and BMI1, the RNA pull-down assay was performed, and then it was found that a positive signal was observed in proteins pulled down with lncKLF6 rather than in samples that were bound to antisense

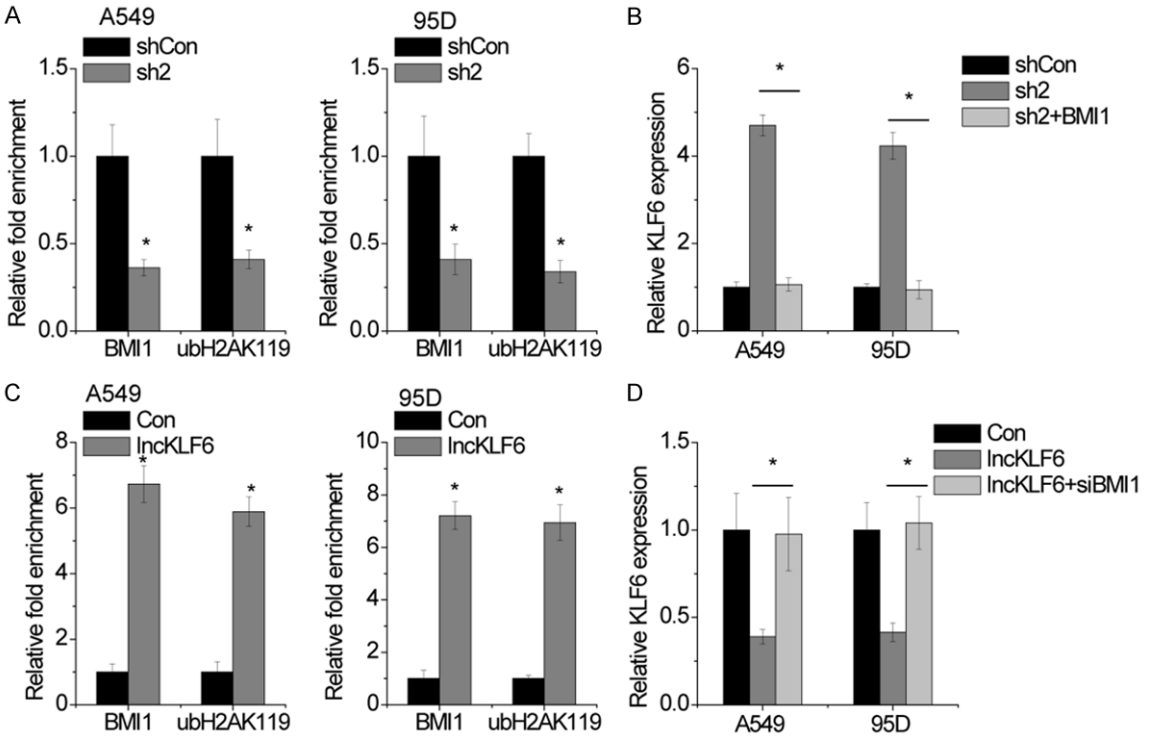


**Figure 4.** lncKLF6 represses KLF6 transcription through H2AK119 ubiquitination. A. The level of BMI1 and ubH2AK119 at the KLF6 promoter in NSCLC cells with lncKLF6 knockdown were analyzed by ChIP. B. The BMI1 overexpression abolished the upregulation of KLF6 expression mediated by lncKLF6 knockdown. C. The level of BMI1 and ubH2AK119 at the KLF6 promoter in NSCLC cells with lncKLF6 overexpression were analyzed by ChIP. D. Depletion of BMI1 rescued the KLF6 expression decreased by lncKLF6 overexpression. *P<0.05.

lncKLF6 (**Figure 2E**). A series of deletion-mapping analyses also showed that a 710 nt region in the 5' end of the lncKLF6 RNA was essential for the lncKLF6-BMI1 interaction (**Figure 2F**). Generally, these results demonstrate that lncKLF6 associates with BMI1.

Next, we explored the regulatory relationship between lncKLF6 and BMI1. lncRNA could modulate the protein stability of its interacting proteins. Intriguingly, silencing lncKLF6 significantly decreased the protein level of BMI1 (**Figure 2G**). To further explore the mechanism of lncKLF6-mediated BMI1 regulation, we treated A549 cells with cycloheximide (CHX), the inhibitor of protein synthesis, and detected the protein levels of BMI1 in different time points through the western blot. Then, it was observed that lncKLF6 knockdown significantly shortened the half-life of BMI1 (**Figure 2H**). In agreement with this observation, the BMI1 expression decreased when the lncKLF6 shRNA reached a level that was comparable to that in control cells by the treatment of proteasome degradation inhibitor MG132 (**Figure 2H**). Moreover, the levels of polyubiquitin-modified BMI1 in lncKLF6 knockdown cells were examined. Higher BMI ubiquitination levels were also observed in lncKLF6 knockdown cells when treated with MG132 (**Figure 2I**).

*lncKLF6 stabilizes BMI1 through recruiting deubiquitinase USP22*

We further explored the underlying mechanism, by which lncKLF6 increased BMI1 protein stability. Previous study demonstrated that deubiquitinase USP22 stabilized BMI1 [18]. Our results of RNA pull-down assay followed by mass spectrometry analysis showed that lncKLF6 also interacted with USP22. It was speculated that lncKLF6 stabilized BMI1 protein through recruiting USP22. Then, the RIP assay was performed to confirm the association between lncKLF6 and USP22. The results displayed that lncKLF6 was significantly enriched by the USP22 antibody when compared with the negative control IgG (**Figure 3A**). Interestingly, the co-IP assay showed that USP22 interacted with BMI1, and their interaction was significantly

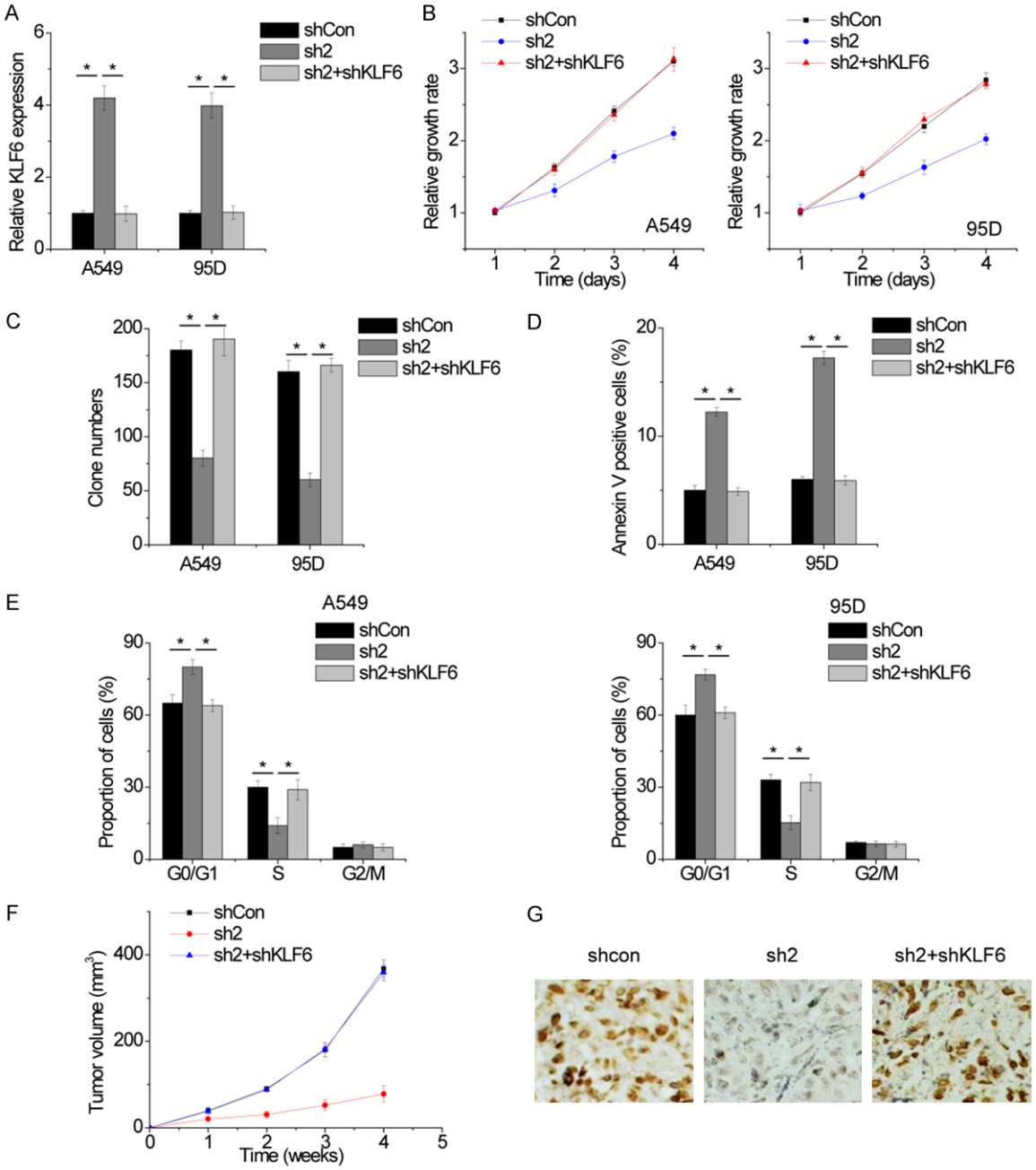


**Figure 5.** lncKLF6 promotes NSCLC growth through suppression of KLF6 transcription *in vitro* and *in vivo*. A. The KLF6 expression was silenced in NSCLC cells with lncKLF6 knockdown. B. The KLF6 knockdown reversed the NSCLC cell proliferation decreased by lncKLF6 knockdown. C. The KLF6 knockdown reversed the NSCLC clone formation reduced by lncKLF6 knockdown. D. The apoptosis rate of NSCLC cells were increased after lncKLF6 knockdown, while reversed by KLF6 knockdown. E. The cell cycle distribution of indicated NSCLC cells was analyzed by flow cytometry. F. The indicated A549 cell clones were subcutaneously injected into the right flanks of male BALB/c nude mice. The tumor volume was measured. G. Immunohistochemical staining of Ki67 in xenograft tumor tissues, which indicated the degree of tumor proliferation. *P<0.05.

decreased by lncKLF6 knockdown (**Figure 3B**). We transfected USP22 siRNAs into NSCLC cells with lncKLF6 overexpression, and found that the depletion of USP22 attenuated the increase of BMI1 induced by lncKLF6 overexpression (**Figure 3C**). In addition, USP22 knockdown led to an increase in the BMI1 ubiquitination level in A549 cells with lncKLF6 overexpression (**Figure 3D**). Collectively, USP22 is involved in lncKLF6-mediated BMI1 upregulation.

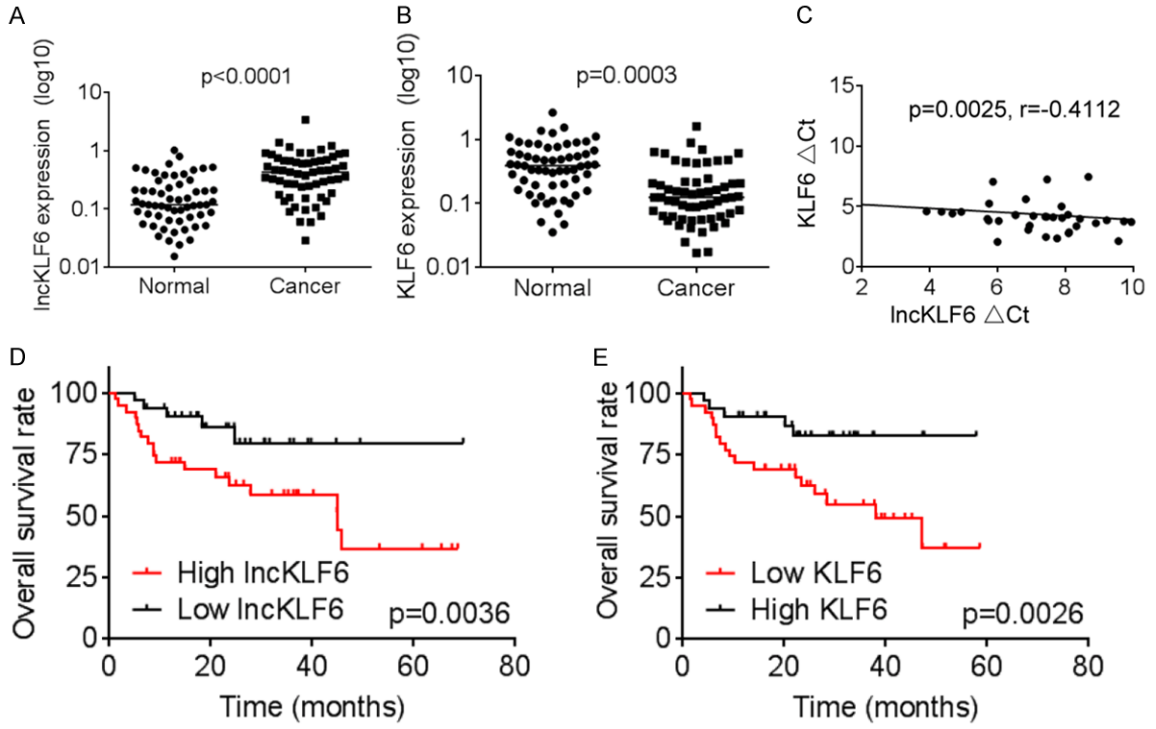


**Figure 6.** lncKLF6 is upregulated in NSCLC and negatively correlates with KLF6 expression. A. The lncKLF6 expression in 59 pairs of NSCLC and matched normal tissues was determined by qRT-PCR. B. The KLF6 expression in 59 pairs of NSCLC and matched normal tissues was determined by qRT-PCR. C. The correlation between lncKLF6 and KLF6 expression in NSCLC tissues. D. The relationship between lncKLF6 and prognosis of NSCLC patients. E. The relationship between KLF6 and prognosis of NSCLC patients.

*lncKLF6 represses KLF6 transcription through H2AK119 ubiquitination*

Given that BMI1 represses transcription via binding to the promoter region and ubiquitinating H2A at K119 (ubH2AK119) of target genes, we then detected whether lncKLF6 had an effect on the levels of BMI1 binding and ubH2AK119 at the *KLF6* promoter by the ChIP assay followed by qRT-PCR. Then, it was found that lncKLF6 knockdown decreased the occupancy of BMI1 and ubiquitination levels of H2AK119 in the *KLF6* promoter (**Figure 4A**). In addition, restoring BMI1 expression abolished the upregulation of KLF6 expression mediated by lncKLF6 knockdown (**Figure 4B**). Conversely, the overexpression of lncKLF6 increased the occupancy of BMI1 and ubH2AK119 levels in KLF6 promoter regions (**Figure 4C**). The depletion of BMI1 rescued the KLF6 expression suppressed by lncKLF6 overexpression (**Figure 4D**). To sum up, these observations demonstrated that lncKLF6 inhibited KLF6 expression through regulating BMI1 protein stability.

*lncKLF6 promotes NSCLC growth through the suppression of KLF6 in vitro and in vivo*

To determine the functional significance of the regulation concerning KLF6 by lncKLF6, we knockdown the KLF6 expression in lncKLF6-silencing cells (**Figure 5A**). CCK-8 and colony formation assays showed that the knockdown of lncKLF6 significantly suppressed the cellular proliferation of both A549 and 95D cells, whereas the downregulation of KLF6 rescued this decrease (**Figure 5B** and **5C**). In addition, lncKLF6 knockdown resulted in the increased percentage of apoptotic cells and cell cycle arrest, while the depletion of KLF6 eliminated these effects (**Figure 5D** and **5E**).

To show the importance of lncKLF6 in regulating the growth of NSCLC cells, we performed *in vivo* tumor growth assays. Compared with the control groups, mice injected with lncKLF6-silencing A549 cells displayed a significant inhibition of tumor growth. KLF6 knockdown was able to counteract the suppressive activities of lncKLF6 knockdown (**Figure 5F**). In addition,

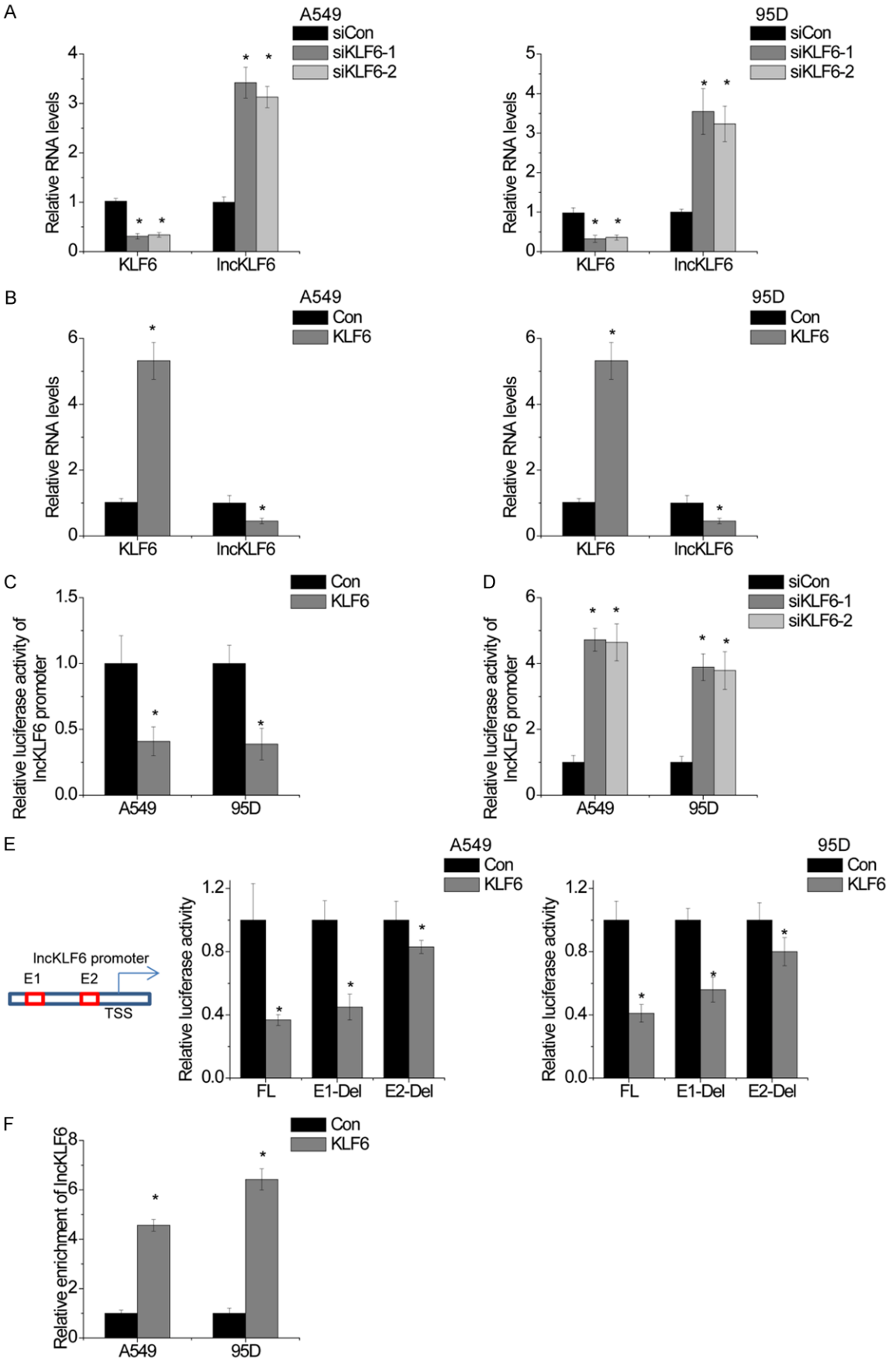
A
A549
siCon
siKLF6-1
siKLF6-2
Relative RNA levels
KLF6
lncKLF6
95D
B
Con
KLF6
C
Relative luciferase activity of lncKLF6 promoter
A549
95D
D
E
lncKLF6 promoter
E1
E2
TSS
Relative luciferase activity
FL
E1-Del
E2-Del
F
Relative enrichment of lncKLF6

**Figure 7.** KLF6 suppresses lncKLF6 transcription. A. A549 and 95D cells were tranfected with KLF6 siRNAs. The expression levels of KLF6 and lncKLF6 were detected by qRT-PCR. B. A549 and 95D cells were tranfected with KLF6. The expression levels of KLF6 and lncKLF6 were detected by qRT-PCR. C. A549 and 95D cells were tranfected with KLF6. The relative luciferase activity of *lncKLF6* promoter was detected by luciferase assay. D. A549 and 95D cells were tranfected with KLF6 siRNAs. The relative luciferase activity of *lncKLF6* promoter was detected by luciferase assay. E. Two predicted KLF6-binding sites of the *lncKLF6* promoter was individually deleted and named E1-Del and E2-Del. Luciferase assay was employed to detect transcriptional activities of the two *lncKLF6* promoter deletion mutants when KLF6 expression was enforced in NSCLC cells. F. ChIP assays showed that KLF6 bound to the E2 element of *lncKLF6* promoter. IgG served as a negative control. *P<0.05.

immunohistochemical (IHC) staining revealed the reduction of the proliferation marker Ki67 expression in the xenograft tumours grown from lncKLF6 knockdown A549 cells, and the downregulation of KLF6 attenuated this decrease (**Figure 5G**). Taken together, these results demonstrated an important role of KLF6 in lncKLF6-induced growth.

*lncKLF6 is upregulated in NSCLC and negatively correlates with KLF6 expression*

To further determine whether the lncKLF6-mediated suppression of KLF6 expression is clinically relevant to NSCLC development, qRT-PCR was performed to detect the expression patterns of lncKLF6 and KLF6, and their correlation in 70 pairs of NSCLC tissue and matched non-tumor tissue was analyzed. As shown in **Figure 6A** and **6B**, lncKLF6 was significantly increased, while KLF6 was markedly decreased in NSCLC tissue than that in matched non-tumor tissue. Moreover, a negative correlation between lncKLF6 and KLF6 expression in NSCLC tissue was observed (**Figure 6C**).

Furthermore, we examined the relationship between the expression of lncKLF6 and KLF6 and the clinical outcome of NSCLC patients. The results showed that the NSCLC patients with high lncKLF6 or low KLF6 expression had significantly decreased overall survival (**Figure 6D** and **6E**). Taken together, our results suggest that lncKLF6 and KLF6 expression may function as prognostic predictors for NSCLC patients.

*KLF6 suppresses lncKLF6 transcription*

Interestingly, bioinformatics analysis predicted that KLF6 may bind to the promoter region of the *lncKLF6* gene. It was confirmed whether KLF6 could regulate lncKLF6 expression in turn. The results of the qRT-PCR assay demonstrated that the deletion of KLF6 by siRNAs significantly upregulated lncKLF6 expression (**Figure 7A**), whereas the transfection of KLF6 inhibited lncKLF6 expression (**Figure 7B**). Furthermore, the luciferase assay showed that the activity of the *lncKLF6* promoter was significantly suppressed by KLF6 overexpression (**Figure 7C**), while increased by KLF6 knockdown (**Figure 7D**). To further clarify which element was critical for the suppression of lncKLF6 mediated by KLF6, the two predicted KLF6-binding sites were individually deleted. Then, it was found that KLF6 only slightly inhibited lncKLF6 transcription activities without the E2 element (**Figure 7E**), indicating that the E2 element was essential for KLF6 to inactivate lncKLF6 transcription. To further confirm these results, the ChIP assay was performed with the anti-KLF6 antibody, followed by qRT-PCR detection with the specific primer for the E2 element. As shown in **Figure 7F**, KLF6 could significantly bind to the *lncKLF6* promoter. These findings suggest that there is a regulatory feedback loop between lncKLF6 and KLF6 expression in NSCLC cells.

## Discussion

In the current study, we identified a novel functional lncRNA lncKLF6, which repressed the nearby protein-coding gene KLF6 expression. lncKLF6 recruited USP22 to stabilize BMI1 protein and then suppressed KLF6 transcription. Moreover, it was found that lncKLF6 enhanced cell proliferation and inhibited cell apoptosis in a KLF6-dependent manner. The upregulation of lncKLF6 and downregulation of KLF6 also predicted the poor prognosis of NSCLC patients. Meanwhile, lncKLF6 transcription was inhibited by KLF6. Thus, our results indicated that lncKLF6 acts as an oncogene in NSCLC and may be taken as a promising therapeutic target for NSCLC.

Recent studies have suggested that lncRNAs are important regulators for many biological processes, more than 'transcriptional noise' [19]. lncRNAs play important roles in tumori-

genesis and metastasis through association with other molecules including microRNAs, proteins and mRNAs [4, 20]. Previous study demonstrated that intergenic lncRNAs function *in cis* to regulate the transcription of neighboring genes *in trans* [14]. For example, lncTCF7 promoted its nearby protein-coding gene *TCF7* expression through recruiting three core subunits of the SWI/SNF complex, BRG1, BAF170, and SNF5, to promote cancer stem cells' self-renewal and tumor propagation [21]. In the present study, it is found that lncKLF6 also suppresses its neighboring gene *KLF6* transcription. Mechanistically, lncKLF6 was mainly localized in the nuclei and interacted with BMI1. lncKLF6 was associated with USP22 to suppress the ubiquitination level of BMI1 and increase the ubH2AK119 level, and subsequently inhibited KLF6 expression. Previous study reported that USP22 could interact with BMI1 and increased its stability [18]. Interestingly, it was revealed that the knockdown of lncKLF6 significantly suppressed the interaction between BMI1 and USP22, indicating that lncKFL6 functions as a scaffold for the BMI1-USP22 association.

KLF6 is a putative tumor suppressor in some cancers, including NSCLC. The loss of KLF6 expression is also correlated with cancer progression, tumor recurrence, and short survival time [22, 23]. It has been reported that KLF6 is downregulated in primary NSCLC tissue, and overexpression KLF6 induced cell apoptosis and suppressed cell proliferation [15]. KLF6 promoted G1 cell cycle arrest mainly through CDKN1A promoter transactivation [24]. In addition, KLF6 transactivated multiple genes that inhibited the NF-κB pathway and consequently reduced NF-κB nuclear localization and downregulated NF-κB targets [25]. The abnormal epigenetic alteration of KLF6 has also been detected [26]. However, whether lncRNAs are involved in the loss of KLF6 remains unclear. Our study demonstrated that lncKLF6 worked as the upstream of KLF6. lncKLF6 epigenetically silenced KLF6 expression through associating with BMI1 and increasing the ubiquitination of H2AK119. Intriguingly, it was found that KLF6 directly bounded to the lncKLF6 promoter region and suppressed its transcription. Thus, these findings suggest that the lncKLF6-BMI1-KLF6 axis may serve as a potential target for NSCLC therapies.

In conclusion, it is demonstrated that lncRNA lncKLF6 inhibits the KLF6 expression through BMI1 and USP22 and consequently promotes NSCLC growth. Our study also presents that the lncRNA regulates the nearby protein-coding genes and provides insight into the complex biological functions of lncRNAs.

## Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Fei Li, Department of Pulmonary and Critical Care Medicine (PCCM) ward II, Hebei Cangzhou Central Hospital, Cangzhou 061000, Hebei Province, China. E-mail: lifeicz@hainan.net

## References

[1] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015; 65: 87-108.

[2] Hu X, Feng Y, Zhang D, Zhao SD, Hu Z, Greshock J, Zhang Y, Yang L, Zhong X, Wang LP, Jean S, Li C, Huang Q, Katsaros D, Montone KT, Tanyi JL, Lu Y, Boyd J, Nathanson KL, Li H, Mills GB and Zhang L. A functional genomic approach identifies FAL1 as an oncogenic long noncoding RNA that associates with BMI1 and represses p21 expression in cancer. Cancer Cell 2014; 26: 344-357.

[3] Torre LA, Siegel RL and Jemal A. Lung cancer statistics. Adv Exp Med Biol 2016; 893: 1-19.

[4] Peng Z, Liu C and Wu M. New insights into long noncoding RNAs and their roles in glioma. Mol Cancer 2018; 17: 61.

[5] Morlando M and Fatica A. Alteration of epigenetic regulation by long noncoding RNAs in cancer. Int J Mol Sci 2018; 19.

[6] Xue M, Chen LY, Wang WJ, Su TT, Shi LH, Wang L, Zhang W, Si JM, Wang LJ and Chen SJ. HOTAIR induces the ubiquitination of Runx3 by interacting with Mex3b and enhances the invasion of gastric cancer cells. Gastric Cancer 2018; 21: 756-764.

[7] Liu M, Jia J, Wang X, Liu Y, Wang C and Fan R. Long non-coding RNA HOTAIR promotes cervical cancer progression through regulating BCL2 via targeting miR-143-3p. Cancer Biol Ther 2018; 19: 391-399.

[8] Liu YW, Sun M, Xia R, Zhang EB, Liu XH, Zhang ZH, Xu TP, De W, Liu BR and Wang ZX. LincHOTAIR epigenetically silences miR34a by binding to PRC2 to promote the epithelial-to-mesenchymal transition in human gastric cancer. Cell Death Dis 2015; 6: e1802.

[9] Li J, Li Z, Zheng W, Li X, Wang Z, Cui Y and Jiang X. PANDAR: a pivotal cancer-related long noncoding RNA in human cancers. Mol Biosyst 2017; 13: 2195-2201.
[10] Ricciuti B, Mencaroni C, Paglialunga L, Paciullo F, Crino L, Chiari R and Metro G. Long noncoding RNAs: new insights into non-small cell lung cancer biology, diagnosis and therapy. Med Oncol 2016; 33: 18.
[11] Li Y, Yang Y, Li J, Liu H, Chen F, Li B, Cui B and Liu Y. USP22 drives colorectal cancer invasion and metastasis via epithelial-mesenchymal transition by activating AP4. Oncotarget 2017; 8: 32683-32695.
[12] Zhang J, Luo N, Tian Y, Li J, Yang X, Yin H, Xiao C, Sheng J, Li Y, Tang B and Li R. USP22 knockdown enhanced chemosensitivity of hepatocellular carcinoma cells to 5-Fu by up-regulation of Smad4 and suppression of Akt. Oncotarget 2017; 8: 24728-24740.
[13] Yang F, Zhang L, Huo XS, Yuan JH, Xu D, Yuan SX, Zhu N, Zhou WP, Yang GS, Wang YZ, Shang JL, Gao CF, Zhang FR, Wang F and Sun SH. Long noncoding RNA high expression in hepatocellular carcinoma facilitates tumor growth through enhancer of zeste homolog 2 in humans. Hepatology 2011; 54: 1679-1689.
[14] Rinn JL and Chang HY. Genome regulation by long noncoding RNAs. Annu Rev Biochem 2012; 81: 145-166.
[15] Ito G, Uchiyama M, Kondo M, Mori S, Usami N, Maeda O, Kawabe T, Hasegawa Y, Shimokata K and Sekido Y. Kruppel-like factor 6 is frequently down-regulated and induces apoptosis in non-small cell lung cancer cells. Cancer Res 2004; 64: 3838-3843.
[16] Gao Y, Li H, Ma X, Fan Y, Ni D, Zhang Y, Huang Q, Liu K, Li X, Wang L, Gu L, Yao Y, Ai Q, Du Q, Song E and Zhang X. KLF6 suppresses metastasis of clear cell renal cell carcinoma via transcriptional repression of E2F1. Cancer Res 2017; 77: 330-342.
[17] Long Y, Wang X, Youmans DT and Cech TR. How do lncRNAs regulate transcription? Sci Adv 2017; 3: eaao2110.
[18] Ma Y, Fu HL, Wang Z, Huang H, Ni J, Song J, Xia Y, Jin WL and Cui DX. USP22 maintains gastric cancer stem cell stemness and promotes gastric cancer progression by stabilizing BMI1 protein. Oncotarget 2017; 8: 33329-33342.
[19] Louro R, Smirnova AS and Verjovski-Almeida S. Long intronic noncoding RNA transcription: expression noise or expression choice? Genomics 2009; 93: 291-298.
[20] Yang L, Tang Y, Xiong F, He Y, Wei F, Zhang S, Guo C, Xiang B, Zhou M, Xie N, Li X, Li Y, Li G, Xiong W and Zeng Z. LncRNAs regulate cancer metastasis via binding to functional proteins. Oncotarget 2018; 9: 1426-1443.
[21] Wang Y, He L, Du Y, Zhu P, Huang G, Luo J, Yan X, Ye B, Li C, Xia P, Zhang G, Tian Y, Chen R and Fan Z. The long noncoding RNA lncTCF7 promotes self-renewal of human liver cancer stem cells through activation of Wnt signaling. Cell Stem Cell 2015; 16: 413-425.
[22] Hsu LS, Huang RH, Lai HW, Hsu HT, Sung WW, Hsieh MJ, Wu CY, Lin YM, Chen MK, Lo YS and Chen CJ. KLF6 inhibited oral cancer migration and invasion via downregulation of mesenchymal markers and inhibition of MMP-9 activities. Int J Med Sci 2017; 14: 530-535.
[23] He AD, Xie W, Song W, Ma YY, Liu G, Liang ML, Da XW, Yao GQ, Zhang BX, Gao CJ, Xiang JZ and Ming ZY. Platelet releasates promote the proliferation of hepatocellular carcinoma cells by suppressing the expression of KLF6. Sci Rep 2017; 7: 3989.
[24] Kimmelman AC, Qiao RF, Narla G, Banno A, Lau N, Bos PD, Nunez Rodriguez N, Liang BC, Guha A, Martignetti JA, Friedman SL and Chan AM. Suppression of glioblastoma tumorigenicity by the Kruppel-like transcription factor KLF6. Oncogene 2004; 23: 5077-5083.
[25] Masilamani AP, Ferrarese R, Kling E, Thudi NK, Kim H, Scholtens DM, Dai F, Hadler M, Unterkircher T, Platania L, Weyerbrock A, Prinz M, Gillespie GY, Harsh GI, Bredel M and Carro MS. KLF6 depletion promotes NF-kappaB signaling in glioblastoma. Oncogene 2017; 36: 3562-3575.
[26] Song J, Kim CJ, Cho YG, Kim SY, Nam SW, Lee SH, Yoo NJ, Lee JY and Park WS. Genetic and epigenetic alterations of the KLF6 gene in hepatocellular carcinoma. J Gastroenterol Hepatol 2006; 21: 1286-1289.