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BAP18 induces growth of non-small-cell lung carcinoma through upregulating transcriptional level of CCND1/2

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Abstract. – OBJECTIVE: To elucidate the biological function of BAP18 (BPTF-associated protein of 18 kDa) in non-small-cell lung carcinoma (NSCLC) and the molecular mechanism.

PATIENTS AND METHODS: Relative levels of BAP18 in NSCLC tissues were detected by quantitative real-time polymerase chain reaction (qRT-PCR), and its influence on pathological characteristics of NSCLC patients was analyzed. Correlation between BAP18 and Ki67 levels in NSCLC was assessed by Pearson correlation test. Furthermore, Kaplan-Meier curves were depicted for revealing survival difference in NSCLC patients expressing high or low level of BAP18. Relative levels of BAP18, CCND1, CCND2 and CCND3 in A549 and H1299 cells transfected with siBAP18 were determined, as well as colony number. In addition, after knockdown of protein level of BAP18 in A549 and H1299 cells by lentivirus transfection, cell cycle progression was examined. Co-regulation of BAP18 and CCND1/2 on cell growth of NSCLC was finally detected.

RESULTS: BAP18 was upregulated in NSCLC tissues, especially cases with advanced stage (III-IV) or large tumor size (>5 cm). BAP18 was closely linked to tumor size, TNM staging and lymphatic metastasis in NSCLC. Knockdown of BAP18 reduced transcriptional levels of CCND1 and CCND2 in A549 and H1299 cells. Furthermore, knockdown of BAP18 delayed transition from G1 to S phase, and weakened growth of NSCLC cells.

CONCLUSIONS: BAP18 triggers the progression of NSCLC by regulating transcriptional activities of CCND1/2, which may be a potential target for the treatment and diagnosis of NSCLC.

Key Words:
BAP18, CCND1, CCND2, NSCLC.

Introduction

Lung carcinoma is the main cause of tumor death[1]. According to the classification of histological subtypes, non-small-cell lung carcinoma (NSCLC) exceeds 80%-85% of total lung carcinoma cases, and small-cell lung carcinoma (SCLC) accounts for 15%-20%[2]. Despite encouraging progress in diagnosis and treatment of NSCLC has been made in recent years, the long-term prognosis of NSCLC remains unsatisfactory. The 5-year survival of advanced NSCLC is only about 2%[3,4]. Therefore, clarifying NSCLC pathogenesis contributes enhance therapeutic efficacy.

Cyclin-Ds constitute three cell cycle proteins, that is, Cyclin D1, D2 and D3. They are responsible for controlling cell cycle progression and mitotic growth[5-7]. It has proven that Cyclin D1 is the rate limitation gene of G1/S phase transition[8]. A growing number of evidences have confirmed the vital function of CCND1 in human cancers, including gastric cancer, glioma, renal cell cancer, lung adenocarcinoma and osteosarcoma[9-13]. In addition, CCND2 has been previously reported as a cancer regulator[14-16]. As regulatory subunits of CDK4 or CDK6, CCND1 and CCND2 are required for G1/S phase transition in the cell cycle progression. They are therefore considered to be proto-oncogenes and can be utilized as therapeutic targets for cancers[17].

BAP18 (BPTF-associated protein of 18 kDa), also known as MGC49942, is encoded by C17rf49. BAP18 is a component of chromatin complexes MLL1-WDR5[18] and NURF/BPTF[19].

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Very recently, BAP18 has been found to aggravate the progression of prostate cancer[20] and oral squamous cell carcinoma[21]. In this paper, we aim to explore the biological function of BAP18 in NSCLC and its regulatory effects on CCND1/2. Our findings provide novel ideas for targeted therapy of NSCLC.

Patients and Methods

Sample Collection

NSCLC and paracancerous tissues were collected in 50 patients undergoing surgical resection with complete clinical data and follow-up data between December 2017 and December 2019, and they were immediately frozen and stored at -80°C. The inclusion criteria were as below: patients were clinically diagnosed as NSCLC; No treatment-related contraindications. The exclusion criteria were as below: patients had other therapies such as chemotherapy or radiation. This study was approved by the research Ethics Committee of The Fourth Hospital of Hebei Medical University. Informed consent from patients was obtained before sample collection.

Cell Culture

A549 and H1299 cells were provided by China Center for Type Culture Collection (CCTCC) (Wuhan, China), and cultivated in Roswell Park Memorial Institute-1640 (RPMI-1640) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) at 37°C with 5% CO_2.

Transfection of siRNA and Lentivirus

Transfection of siRNAs was conducted using jet-PRIME (Polyplus, Illkirch, France). Lentiviruses were provided by GeneChem (Shanghai, China). The pcDNA3-FLAG vector and BAP18 cDNA were used to construct FLAG-BAP18 (Table I).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cellular or tissue RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) was used for qRT-PCR. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. BAP18: forward: 5'-TGGCATCTGGTGTCTTGTCA-3', reverse: 5'-TTGGCATCGGAGTCGTTCA-3'; CCND1: forward: 5'-CTGATTGGACAGGCATGGGT-3', reverse: 5'-GTGCCTGGAAGTCAACGGTA- 3'; CCND2: forward: 5'-GCTGTGCATTTACACCGACAA-3', reverse: 5'-GCTCAGTCAGGGCATCACAA-3'; CCND3: forward: 5'-CCCTGGAGAGGCCCTCTGGA-3', reverse: 5'-TTCCAAGAAGCCAAAGCCA-3'; GAPDH forward: 5'-GGAGCGAGATCCCTCCAAAAT-3', reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'; KI67 forward: 5'-ACGCCTGGTTACTATCAAAAGG-3', reverse: 5'-CAGACCCATTTACTTGTGTTGGA-3'.

Colony Formation Assay

Cells were inoculated in the 6-well plate with 1×10^3 cells/well. Medium was replaced once a week in the first week, and twice in the second week. Visible colonies containing more than 50 cells were washed by phosphate-buffered saline (PBS), fixed in methanol for 20 min and dyed with 0.1% crystal violet for 20 min. Colonies were captured and calculated for plotting cell growth rate.

Western Blot

Cells were lysed in radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) on ice for 30 min, and centrifuged at 4°C, 14000×g for 15 min. The concentration of cellular protein was determined by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and loaded on polyvinylidene difluoride (PVDF)

Table I. siRNA duplexes.

siRNA	sense (5′-3′)	antisense (5′-3′)
BAP18	GGGACGAUCUUAAUCACAUdTdT	AUGUGAUUAAGAUCGUCCCdTdT
CCND1	CCACAGCUGUGAAGUUCAUdTdT	AUGAACUUCACAUCUGUGGdTdT
CCND2	CUCAUGACUUCAUUGAGCAdTdT	UGCUCAAUGAAGUCAUGAGdTdT

dT: DNA bases within RNA oligos.

membrane (Millipore, Billerica, MA, USA). The membrane was cut into pieces according to the molecular size and blocked in 5% skim milk for 2 h. They were incubated with primary and secondary antibodies, followed by band exposure using enhanced chemiluminescence (ECL).

Flow Cytometry

Cells were seeded in a 24-well with 1×10^5 per well and fixed in 70% cold ethanol for 2 h. Later, cells were incubated in 100 µL of RNase at 37°C for 30 min, followed by induction of 5 µL of AnnexinV-FITC and 5 µL of propidium iodide (PI) at room temperature in the dark. Cell cycle progression was determined using flow cytometry (FACScan, BD Biosciences, Franklin Lakes, NJ, USA) in triplicate.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA) and Graphpad Prism (Version X; La Jolla, CA, USA) were used for data analysis. Data were expressed as mean±SD (standard deviation). Statistically significant differences between the groups were estimated by the Student's *t*-test. The relationship between BAP18 and pathological parameters in NSCLC patients was determined by χ^2-test. Kaplan-Meier method and log-rank test were introduced for survival analysis. $p<0.05$ considered the difference was statistically significant.

Results

Upregulation of BAP18 in NSCLC

To assess the expression pattern of BAP18 in NSCLC, we collected 50 pairs of NSCLC and paracancerous tissues. Compared with normal tissues, BAP18 was highly expressed in NSCLC tissues (Figure 1A). Classified by TNM staging, BAP18 level was higher in stage III-IV NSCLC cases than that of stage I-II cases (Figure 1B). Higher level of BAP18 was detected

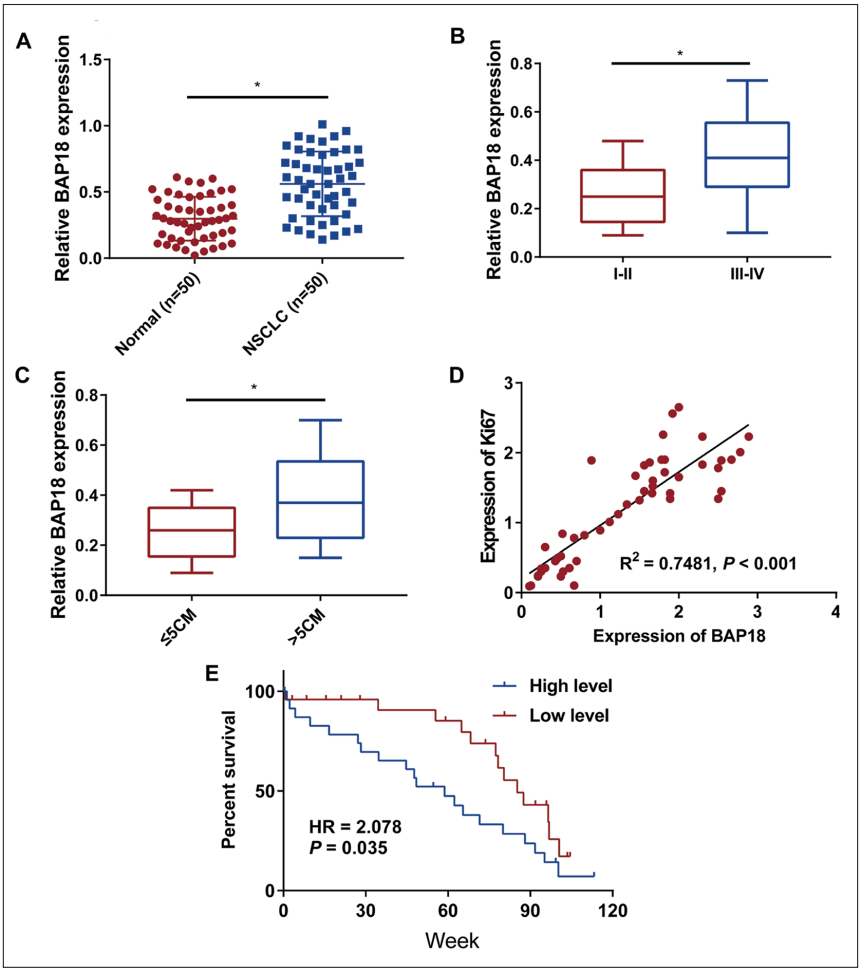

Figure 1. Upregulation of BAP18 in NSCLC. **A,** Higher level of BAP18 in NSCLC tissues than paracancerous ones; **B**, Higher level of BAP18 in stage III-IV NSCLC cases than stage I-II cases; **C,** Higher level of BAP18 in NSCLC tissues with a larger tumor size (>5 cm) than smaller ones (≤5 cm); **D,** BAP18 level was positively correlated to Ki67 in NSCLC tissues; **E,** High level of BAP18 predicted poor prognosis in NSCLC patients.

in NSCLC tissues with a larger tumor size (>5 cm) than that of smaller ones (≤5 cm) (Figure 1C). We thereafter analyzed the correlation between BAP18 and pathological parameters of NSCLC. It is found that BAP18 was correlated to tumor size, TNM staging and lymphatic metastasis status in NSCLC patients (Table II). Ki67 is a well-known proliferation indicator for assessing proliferative ability of tumors. To determine the correlation between BAP18 and proliferative ability of NSCLC, Pearson correlation test was conducted. The data showed a positive correlation between BAP18 and Ki67 levels in NSCLC tissues (Figure 1D). Furthermore, Kaplan-Meier method uncovered that high level of BAP18 was unfavorable to the survival of NSCLC (Figure 1E).

BAP18 Regulated CCNDs in NSCLC Cells

To elucidate the biological effect of BAP18 on NSCLC cell phenotypes, A549 and H1299 cells were transfected with three lines of BAP18 siRNAs (siBAP18 #1, siBAP18 #2 and siBAP18 #3), respectively. Among them, transfection of siBAP18 #1 or siBAP18 #3 could effectively downregulate BAP18, showing a great transfection efficacy (Figure 2A, 2C). Transfection of either siBAP18 #1 or siBAP18 #3 remarkably downregulated mRNA levels of CCND1 and CCND2 in A549 and H1299 cells, whereas CCND3 was not influenced by knockdown of BAP18 (Figure 2B, 2D). It is suggested that BAP18 could regulate transcriptional activities of CCND1/2 in NSCLC cells.

BAP18 Regulated G1/S Transition in NSCLC cells

Cyclin D1 and Cyclin D2 are members of Cyclin-Ds that are responsible for regulating cell cycle progression[23]. Here, A549 and H1299 cells were subjected to lentivirus transfection of sh-BAP18 or sh-NC. Western blot analysis showed decreased protein level of BAP18 after transfection of sh-BAP18, confirming the transfection efficacy (Figure 3A). Flow cytometry data revealed that transfection of sh-BAP18 markedly delayed transition of G1/S phase, and resulted in cell cycle arrest in G1 phase (Figure 3B). It is concluded that BAP18 could affect cell cycle progression in NSCLC cells.

BAP18 Stimulated Proliferative Ability of NSCLC

Transfection of either siBAP18 #1, siBAP18 #2 or siBAP18 #3 remarkably declined colony numbers in A549 cells, suggesting the attenuated proliferative ability (Figure 4A). We speculated that CCND1/2 may be involved in BAP18-regulated NSCLC proliferation. Interestingly, stimulated cell growth rate in A549 cells transfected with FLAG-BAP18 was further declined by co-transfection of either siCCND1 or siCCND2 (Fig-

Table II. Correlation analysis between BAP18 expression and clinicopathological parameters of NSCLC patients.

Clinicopathologic features	N. of cases	BAP18 expression		*p*-value
		Low (n = 25)	High (n = 25)	
Age (years)				0.774
≤ 60	29	15	14	
> 60	21	10	11	
Gender				0.569
Male	28	13	15	
Female	22	12	10	
Tumor size				0.024*
≤ 5 cm	24	16	8	
> 5 cm	26	9	17	
TNM stage				0.023*
I-II	22	15	7	
III-IV	28	10	18	
Lymph node metastasis				
N0	22	16	6	0.004*
N1-3	28	9	19	
Tumor differentiation				
Poor	20	13	7	0.083
Moderate-Well	30	12	18	

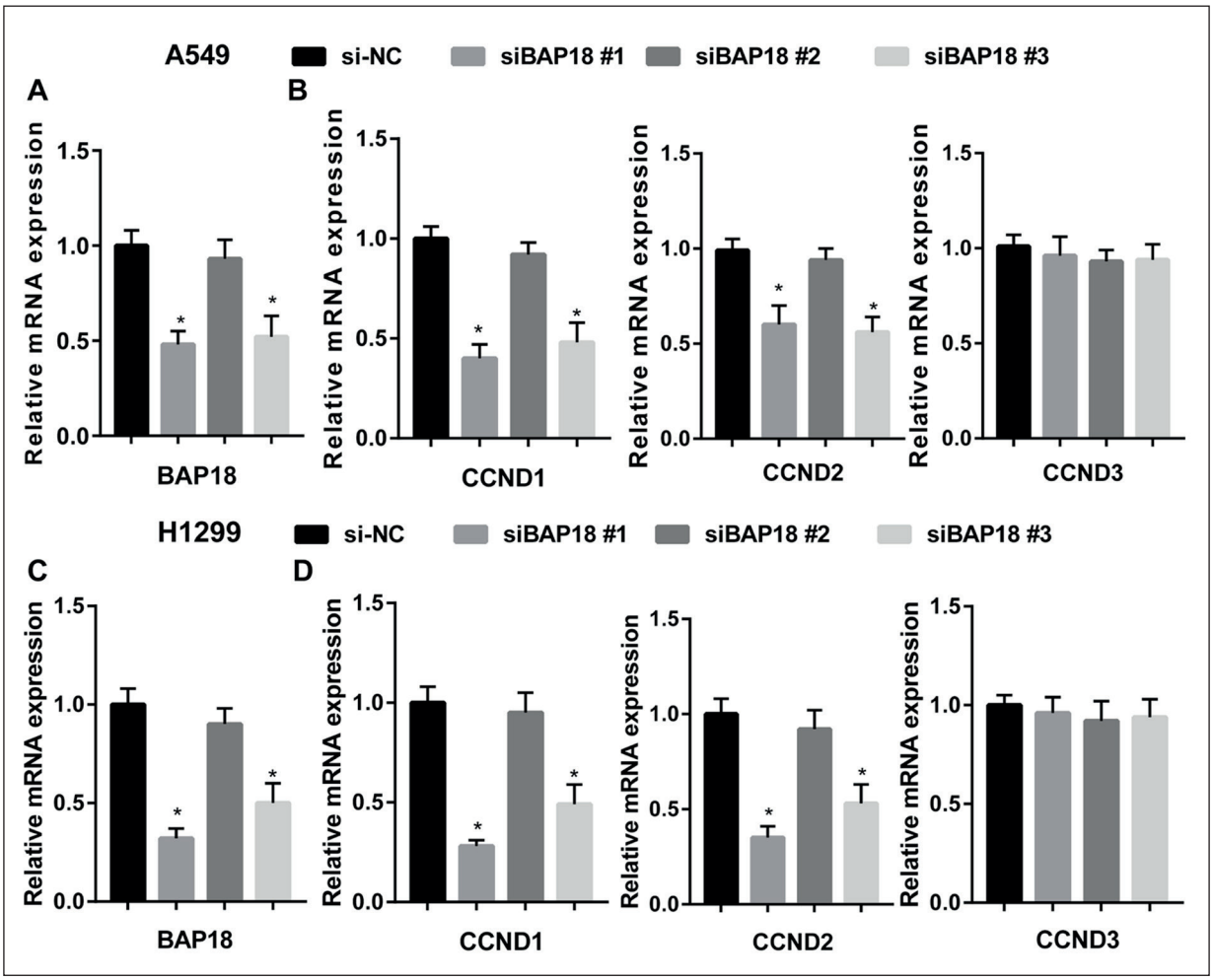

Figure 2. BAP18 regulated CCNDs in NSCLC cells. **A,** Transfection of siBAP18 #1 or siBAP18 #3 significantly downregulated BAP18 in A549 cells; **B,** Knockdown of BAP18 downregulated CCND1 and CCND2 in A549 cells, and CCND3 was not affected; **C,** Transfection of siBAP18 #1 or siBAP18 #3 significantly downregulated BAP18 in H1299 cells; **D,** Knockdown of BAP18 downregulated CCND1 and CCND2 in H1299 cells, and CCND3 was not affected.

ure 4B). Palbociclib is the inhibitor of CDK4/6. We found that application of 25 nM Palbociclib was also capable of reversing the stimulated cell growth rate in A549 cells overexpressing BAP18 (Figure 4C). On the contrary, knockdown of BAP18 declined the cell growth rate, and a similar decreased trend was observed by Palbociclib application (Figure 4D). It is concluded that BAP18 stimulated NSCLC cell growth with the involvement of the CCND1/2-CDKs signaling.

Discussion

BAP18 is considered to be an uncharacterized subunit of the MLL1-MOF complex, which is involved in the upregulation of gene transcription. A SANT domain in the N terminal of BAP18 attributes to the biological function of BAP18 in chromatin remodeling and histone modification[24-26]. As a key subunit of polyhistone methyltransferase complex, BAP18 participates in the regulation of androgen receptor-induced transactivation and thus affects prostate cancer process[20]. A latest report pointed out that BAP18 induces growth of oral squamous cell carcinoma[21]. Our findings demonstrated that BAP18 was upregulated in NSCLC tissues. Knockdown of BAP18 markedly downregulated mRNA levels of CCND1/2 in A549 and H1299 cells. In addition, knockdown of BAP18 was able to inhibit the formation of colonies and cell growth of NSCLC.

NSCLC is a rapidly proliferated malignant tumor with an extremely low rate of 5-year surviv-

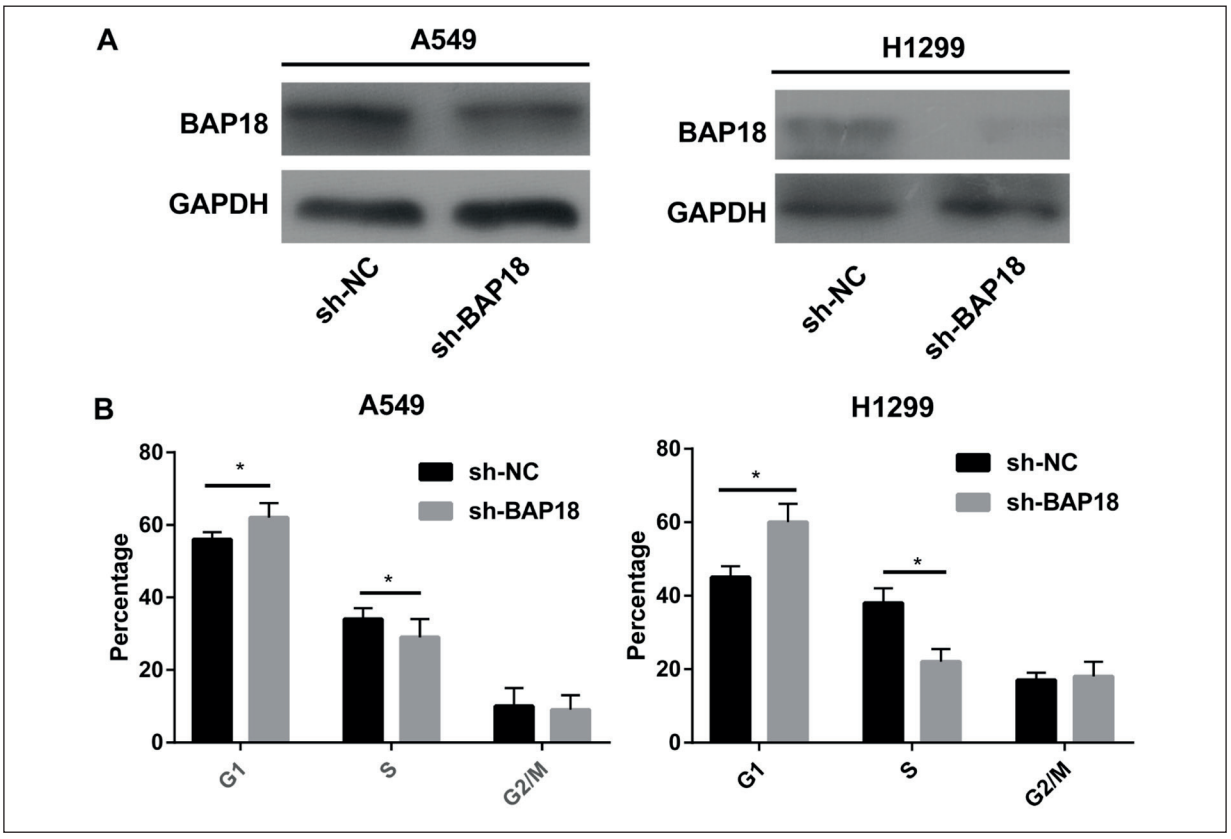

Figure 3. BAP18 regulated G1/S transition in NSCLC cells. **A,** Transfection of sh-BAP18 significantly downregulated protein level of BAP18 in A549 and H1299 cells; **B,** Knockdown of BAP18 inhibited G1/S phase transition in A549 and H1299 cells.

al[1]. Clinical significance of BAP18 in NSCLC was explored. It is found that BAP18 level remained higher in NSCLC cases with advanced stage or larger tumor size. Moreover, Kaplan-Meier method has indicated the prognostic value of BAP18 in NSCLC.

Abnormalities in the transition from G1 to S phase during cell cycle progression are probably risk factors for carcinogenesis[27-29]. Dysfunctional Cyclin D may induce potential carcinogenic reactions, and has a certain impact on tumor prognosis[30]. It is reported that CDK4 or CDK6 can accelerate cell cycle progression *via* phosphorylating substrates, including cell cycle proteins[31]. Palbociclib is a CDK inhibitor with a potential antineoplastic activity, which drives cancer cell senescence through blocking cell cycle progression in G1 phase[32,33]. Interestingly, a recent report[21] showed that BAP18 could promote cell growth in OSCC by upregulating CCND1/2 transcription. In this paper, BAP18 was identified to positively regulate CCND1/2 levels. Knockdown of BAP18 achieved a similar inhibitory effect of Palbociclib on cell growth of NSCLC. Notably, silence of CCND1/2 or Palbociclib application could abolish the role of overexpressed BAP18 in inducing NSCLC proliferation, demonstrating that BAP18 stimulated proliferative ability of NSCLC *via* the CCND1/2-CDKs signaling. The previous research had underlined the role of BAP18 in regulating cell cycle and cell proliferation. However, the role of BAP18 in apoptosis, EMT related assays and *in vivo* studies were not enrolled in this research. In summary, our research had slightly uncovered the function and mechanism of BAP18 in regulating the process of NSCLC, which providing for the target therapy in the future.

Conclusions

BAP18 triggers the progression of NSCLC by regulating transcriptional activities of CCND1/2, which may be a potential target for the treatment and diagnosis of NSCLC.

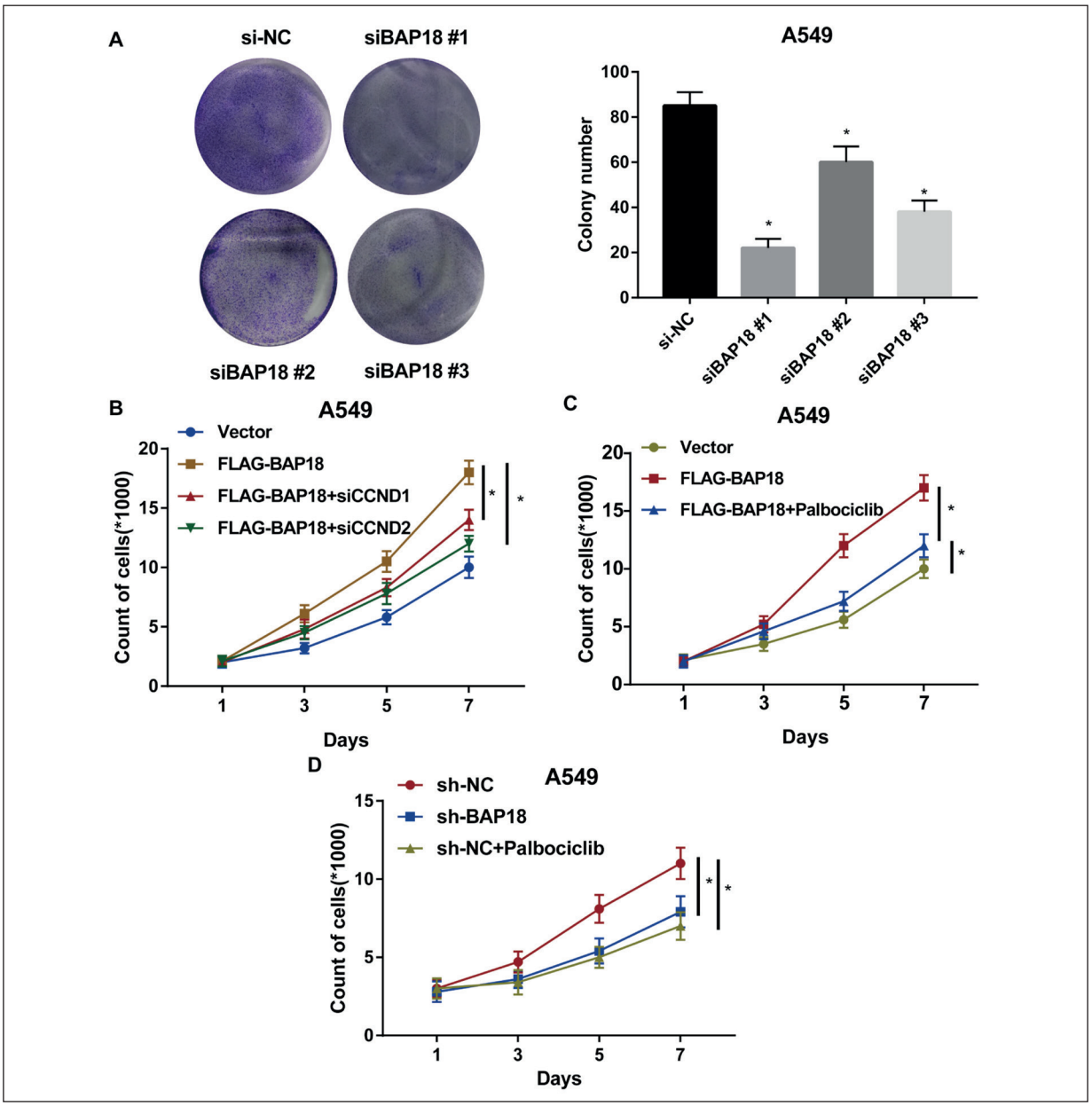

Figure 4. BAP18 stimulated proliferative ability of NSCLC. **A,** Knockdown of BAP18 inhibited colony formation in A549 cells; **B,** Overexpression of BAP18 enhanced cell viability in A549 cells, and knockdown of CCND1/2 inhibited cell growth; **C,** Overexpression of BAP18 enhanced cell viability in A549 cells, and induction of 25 nM Palbociclib inhibited cell growth; **D,** Knockdown of BAP18 or induction of 25 nM Palbociclib inhibited cell viability in A549 cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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