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Nemo-Like Kinase (NLK) Inhibits the Progression of NSCLC via Negatively Modulating WNT Signaling Pathway

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ABSTRACT

Nemo-like kinase (NLK), an evolutionarily conserved serine/threonine kinase, is a critical regulator of various cancers. NLK expression was evaluated by Western blot in 8 paired fresh non-small-cell lung cancer (NSCLC) tissues and immunohistochemistry (IHC) on 83 paraffin-embedded slices. NLK was lowly expressed in NSCLC and significantly associated with NSCLC histological differentiation, clinical stage, lymph node status, and Ki-67. Multivariate analysis indicated that low NLK expression was an independent prognostic factor for NSCLC patients′ low survival rate. In vitro, after the release of NSCLC cell line A549 from serum starvation, the expression of NLK was downregulated, whereas the cell-cycle-related proteins were upregulated. In addition, we used RNA interference to knock down NLK expression, then observed its effects on NSCLC′s growth in vitro. Western blot analyses indicated that deletion of NLK was positively correlated with cell-cycle-related proteins. The present investigation demonstrated that suppression of NLK expression resulted in significant promotion of proliferation in NSCLC cells. And flow cytometry further indicated that loss of NLK promoted cell proliferation by facilitating S-phase and mitotic entry. Besides, the transcription activity of β-catenin/TCF in A549 cells was remarkably enhanced when NLK was knocked down, which suggested that NLK participated in NSCLC cell proliferation via medulating Wnt signaling pathway. Based on these findings, we can provide a potential strategy for NSCLC therapy. J. Cell. Biochem. 115: 81–92, 2014.

KEY WORDS: NON-SMALL-CELL LUNG CANCER; NLK; PROLIFERATION; WNT; PROGNOSIS

Non-small-cell lung cancer (NSCLC) accounts for the most deaths among all types of cancers around the world [Okamoto et al., 2010; Andersen et al., 2011]. The 5-year survival rate of patients suffering from NSCLC is merely 15%, which largely due to the fact that most of them are diagnosed at advanced and later stages. Complete understanding of the molecular mechanisms regarding NSCLC will contribute to the development of diagnostic technologies, new treatment methods and preventive approaches. Genetic factors play important roles in the initiation and progression of NSCLC. Genetic abnormalities in major components of several signaling pathways lead to the occurrence of NSCLC. Recent studies have reported that genetic alterations of tumor associated genes such as p27, β-catenin, and p53 are all involved in these events. However, the investigation on expression alterations of many cancer-related genes has been poorly performed. Thus, increasing the number of molecular markers is urgently needed to clarify the mechanisms and benefit the prognosis of NSCLC. Furthermore, it is still unclear how the molecular variations precisely change the clinical characteristics. Therefore,

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the major molecular events underlying NSCLC remain to be identified.

Nemo-like kinase (NLK), an evolutionarily conserved serine/threonine kinase, is the member of the extracellular signal regulated kinases/microtubule-associated protein kinases (Erks/MAPKs) and cyclin-directed kinases (CDKs) [Brott et al., 1998]. NLK is regulated by the MAPK cascade, which is composed of the MAP kinase kinase kinase (MAPKKK)-type kinase transforming growth factor (TGF)-β-activated kinase (TAK1) [Takada et al., 2007] and the MAP kinase (MAPK)-type kinase NLK [Ishitani et al., 2003]. TAK1 can activate NLK through a TAK1–TAB2–NLK protein kinase cascade, once activated by upstream signals [Takada et al., 2007]. Previous reports indicated that NLK could regulate the activity of diverse transcription factors [Kim et al., 2010], including Smads [Miyazono, 2000], AP-1 [Kamei et al., 1996], p53 [Avantaggiati et al., 1997; Gu et al., 1997], and NF-⊠B [Yasuda et al., 2004], implicating that NLK might play a crucial role in the progression of various cancers. NLK also exerted an essential influence in endoderm induction [Shin et al., 1999], eye and wing development in *Drosophila* [Choi and Benzer, 1994; Zeng et al., 2007], neurogenesis in zebrafish [Ishitani et al., 2010; Ota et al., 2012], as well as oocyte maturation in *Xenopus* [Ishitani et al., 1999; Hyodo-Miura et al., 2002; Ohkawara et al., 2004; Ota et al., 2011], which were related to its role in modulating Wnt/β-catenin or Notch signaling pathways [Ishitani et al., 2010]. NLK acted as a negative regulator in Wnt signaling pathway via phosphorylating T-cell factor/lymphoid enhancer factor (TCF/LEF) proteins, inhibiting the binding of β-catenin/TCF/LEF complex to its transcriptional response elements [Yamaguchi et al., 1995; Shibuya et al., 1996; Hyodo-Miura et al., 2002; Ohkawara et al., 2004; Yamada et al., 2006].

Canonical Wnt/β-catenin signaling is a classical pathway that plays vital roles in organism development and cancer progression [Yamaguchi et al., 1995]. Wnts, secreted proteins involved in a wide range of developmental events, are transduced through binding Frizzled (FZD) family receptors and LRP5/LRP6 co-receptor [Wodarz and Nusse, 1998; Peifer and Polakis, 2000; Katoh, 2007], which leads to subsequent destabilization of axin/GSK-3/APC complex [Zeng et al., 1997; Behrens et al., 1998]. The disassembly of the axin/GSK-3/APC complex abolishes its ability to mediate the phosphorylation and degradation of cytoplasmic β-catenin [Katoh, 2006], β-catenin then accumulates in the nucleus and forms a complex with the TCF/LEF-1 family of high mobility group transcription factors. The β-catenin/TCF/LEF complex activates the transcription of multiple target genes, such as c-myc, cyclin D1, DKK1, Axin2, and survivin. Wnt/β-catenin signaling plays crucial roles in cell proliferation, survival, migration and renewal. The link between Wnt signaling in various types of cancers has been well studied [Lee et al., 1995; Peifer and Polakis, 2000; Katoh, 2006, 2007]. Activating mutations in the components of canonical Wnt signaling have been found in as many as over 90% colorectal cancers [Vasil′ev et al., 1990]. Aberrant activation of Wnt signaling is also frequently observed in liver [Takigawa and Brown, 2008], lung cancers [Mazieres et al., 2005]. Previous reporters have established a strong link between NLK and Wnt activity [Ishitani et al., 1999]. Wnt signal also causes the nuclear entry of TAK1, then activating NLK, which in turn phosphorylates TCFs, thereby downregulating the DNA-binding activity of the TCF-4/β-catenin complex [Smit et al., 2004]. Thus, the activation of TAK1–TAB2–NLK complex forms a feedback loop in preventing hyperactivation of Wnt signaling. Dysregulation of Wnt/β-catenin/NLK signaling is involved in human carcinogenesis, especially in colon [Yasuda et al., 2003] and liver [Yasuda et al., 2004]. NLK expression is decreased in pancreas cancer metastases and induces apoptosis in pancreas cancer cells [Cheng et al., 2012]. Furthermore, NLK over-expression can induce cell growth suppression and apoptosis in DLD-1 human colon cancer cells [Yasuda et al., 2004]. It is also indicated that over-expression of NLK in M1 cells abrogates the ability of c-Myb to maintain the undifferentiated state of these cells [Kanei-Ishii et al., 2004]. However, the expression patterns and clinicopathological significance of NLK in patients with NSCLC is still unclear.

As a negative modulator of the canonical Wnt signaling pathway, the alteration of NLK has been identified in diverse cancers, such as HCC [Jung et al., 2010] and ovarian cancer [Zhang et al., 2011]. So we wonder whether NLK participates in NSCLC. In this study, we aim to investigate the biological role of NLK in NSCLC′s initiation and progression. The expression of NLK in 8 paired tumorous and non-tumorous adjacent specimens and immunohistochemistry (IHC) on 83 patients were assessed. In addition, NLK expression was disrupted by lentivirus-mediated RNA interference to explore its possible role in NSCLC′s progression. Meanwhile, using clone formation, CCK-8 growth curve and flow cytometric assays, we clarified that NLK played an important role in inhibiting NSCLC cell proliferation. Finally, our results found that NLK could negatively regulate the transcription activity and downstream genes of the Wnt signaling pathway. Taken together, we draw the conclusion that NLK is a negative regulator in cell proliferation of NSCLC by modulating the activity of Wnt/β-catenin signaling, and may benefit the diagnosis and therapy of NSCLC.

MATERIALS AND METHODS

PATIENTS AND TISSUE SAMPLES

All the investigations represented in this study were conducted after informed consent was obtained and in accordance with an Institutional Review Board (IRB) protocol approved by the Partners Human Research Committee at the Affiliated Hospital of Nantong University. Eight paired fresh samples were frozen in liquid nitrogen immediately after surgical removal and maintained at −80°C until use for Western blot analysis. All human tissues were collected using protocols approved by the Ethics Committee of Affiliated Hospital of Nantong University. The NSCLC specimens were obtained from 83 patients during 2005 and 2007, who underwent lung resection without preoperative systemic chemotherapy at the Surgery Department of the Affiliated Hospital of Nantong University. The main clinical and pathologic variables are shown in Table I. Forty-seven patients were men while 36 were women, 6 had no significance, and their average age was 60 years (range, 26–77). Tumors were classified as well (grade I; n = 27), moderately (grade II; n = 30), or poorly (grade III; n = 26) differentiated. The follow-up time was 5 years, with a range of 1–52 months. None of the patients received postoperative adjuvant therapy.

TABLE I. NLK and Ki-67 Expression and Clinicopathologic Parameters in 83 NSCLC Specimens

Parameters	Total	NLK expression		P
		Low	High	
Age (year)				0.656
<60	34	19	15	
≥60	49	24	25	
Gender				0.511
Male	47	26	21	
Female	36	17	19	
Tumor size (cm)				0.643
<3	55	27	28	
≥3	28	16	12	
Smoking status				0.377
Yes	36	21	15	
No	47	22	25	
Histological type				0.469
Adenocarcinoma	33	15	18	
Squamous cell carcinoma	39	23	16	
Adenosquamous carcinoma	11	5	6	
Clinical stage				0.001*
I	31	8	23	
II	31	20	11	
III	21	15	6	
Histological differentiation				0.002*
Well	27	7	20	
Mod	30	17	13	
Poor	26	19	7	
Lymph node status				0.015*
0	34	12	22	
>0	49	31	18	
Ki-67 expression				<0.001*
Low	32	4	28	
High	51	39	12	

Note: Statistical analyses were performed by the Pearson χ^2 test.
*$P < 0.05$ was considered significant.

ANTIBODIES

The antibodies used for IHC in this study included: rabbit anti-human NLK polyclonal antibody (Sigma Chemicals, St. Louis, MO) and mouse anti-human Ki-67 monoclonal antibody (Santa Cruz Biotechnology). Antibodies for Western blotting included: rabbit anti-human NLK polyclonal antibody, mouse anti-human PCNA monoclonal antibody, rabbit anti-human cyclin D1 polyclonal antibody, rabbit anti-human cyclin A polyclonal antibody, mouse anti-human c-myc polyclonal antibody, rabbit anti-human GAPDH polyclonal antibody, all the antibodies were purchased from Santa Cruz Biotechnology.

WESTERN BOLT

Tissue and cell protein were immediately homogenized in a homogenization buffer containing 50 mM Tirs–HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 5 mM EDTA, 60 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM NaF, and complete protease inhibitor cocktail (Roche Diagnostics), and then centrifuged at 12,000*g* for 15 min to collect the supernatant. Protein concentrations were measured with a Bio-Rad protein assay (Bio-Rad, Hercules, CA). The supernatant diluted in 2× SDS loading buffer and boiled for 15 min. Proteins were separated with SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to polyvinylidene difluoride filter (PVDF) membranes (Millipore, Bedford, MA). The membranes were blocked with 5% no-fat milk in TBST (150 mM NaCl, 20 mM Tris, 0.05% Tween-20). After 2 h at room temperature, the membranes were washed with TBST for three times and then incubated overnight with the primary antibodies and later horseradish peroxidase-linked IgG as the secondary antibodies. The band density was measured with a computer-assisted image-analysis system (Adobe Systems, San Jose, CA) and normalized against GAPDH levels. Values were responsible for at least three independent reactions.

IMMUNOHISTOCHEMISTRY

Immunostaining was performed using the avidin biotin peroxidase complex. The sections were deparaffinized using a graded ethanol series, and endogenous peroxidase activity was blocked by soaking in 3% hydrogen peroxide for 10 min. And then, the sections were processed in 10 mmol/L citrate buffer (pH = 6.0) and heated to 121°C in an autoclave for 20 min to retrieve the antigen. After rinsing in PBS (pH = 7.2), the sections were then incubated with anti-NLK antibody (diluted 1:1,000; Sigma) for 1 h at room temperature, anti-Ki-67 antibody (diluted 1:400; Santa Cruz Biotechnology) for 2 h at room temperature. Negative control slides were processed in parallel using a non-specific immunoglobulin IgG (Sigma) at the same concentration as the primary antibody. All slides were processed using the peroxidase-anti-peroxidase method (DAKO, Hamburg, Germany). After being rinsed in PBS, the peroxidase reaction was visualized by incubating the sections with the liquid mixture (0.02% diaminobenzidine tetrahydrochloride, 0.1% phosphate buffer solution, and 3% H_2O_2). After rinsing in water, the sections were counterstained with hematoxylin, dehydrated, and cover slipped.

IMMUNOHISTOCHEMICAL EVALUATION

All of the immunostained sections were evaluated in a blinded manner without knowledge of the clinical and pathological parameters of the patients. For assessment of NLK and Ki-67, five high-power fields in each specimen were selected randomly, cytoplasm and nuclear staining were examined. More than 1,000 cells were counted to determine the mean percent, which represented the percentage of immunostained cells relative to the total number of cells. In more than one half of the samples, staining was repeated three times to avoid technical errors, and a consensus was achieved. Three independent pathologists evaluated the immunostaining results. When evaluating the NLK protein immunoreaction, staining intense was classified to be 0 (negatively or poorly staining), 1 (moderately staining), 2 (strongly staining). To allow univariate and multivariate analyses, we divided patients into three groups according to NLK expression ratio (50%, 75%): high expression group (>75%) to be score of 3, moderately expression group (50–75%) to be 2, and low expression group (<50%) to be 1. Then multiply the two scores, and divided patients into two groups according to the scores (average = 3): high expression group (>3), and low expression group (≤3). When evaluating the Ki-67 protein immunoreaction, staining was scored in a semi-quantitative fashion. A cut-off value of 50% or more positively stained nuclei in five high-power fields was used to define Ki-67 staining, high expression group (≥50%) and low expression group (<50%).

CELL CULTURES

The human lung cancer cell line A549 and SK-MES-1 were purchased from the Shanghai Institute of Cell Biology, Academic Sinica. A549

cells were cultured in high-glucose DMEM (GibCo BRL, Grand Island, NY), while SK-MES-1 cells were cultured in high-glucose MEM (Thermo Scientific, China), supplemented with 10% fetal bovine serum, 100 U/ml penicillin–streptomycin mixture (GibCo BRL) at 37°C and 5% CO_2. The medium was replaced 24 h later with fresh medium for transfection.

LENTIVIRUS PACKAGE AND INFECTION

The NLK-shRNA and control-shRNA lentiviruses were obtained from Gene Chem technology. The shRNA target sequences were: 5′-GAATATCCGCTAAGGATGC-3′, and 5′-CAGATCCAAGAGATG-GAAA-3′. A549 and SK-MES-1 cells were infected with control-shRNA or NLK-shRNA lentiviruses according to the manufacture′s protocol.

CELL CYCLE ANALYSES

In order to analyze cell cycle, cells were fixed in 70% ethanol overnight at 4°C and then incubated with 1 mg/ml RNase A for 30 min. Subsequently, cells were stained with 0.5% Tween-20, propidium iodide (PI, 50 mg/ml, Becton Dickinson, San Jose, CA) in PBS, and analyzed using a Becton Dickinson flow cytometer BD FACScan as well as Cell Quest acquisition and analysis programs. Gating was set to exclude cell debris, cell doublets, and cell clumps.

CELL COUNTING KIT (CCK)-8 ASSAY

Cell proliferation was valued using the commercial Cell Counting Kit (CCK)-8 assay in accordance with the manufacturer′s instructions. Briefly, cells were seeded onto 96-well cell culture cluster plates (Corning, Inc., Corning, NY) at a concentration of 2×10^4 cells/well in volumes of 100 μl, and grown overnight. CCK-8 reagents (Dojindo, Kumamoto, Japan) were added to each well under different treatments, incubated for an additional 2 h at 37°C, and the absorbency was measured at a test wavelength of 490 nm and a reference wavelength of 650 nm with a microplate reader (Bio-Rad). All the data were obtained from three independent experiments.

CLONE FORMATION

A549 cells were seeded at 200 cells/well in 6-well culture plates after infected with control-shRNA and NLK-shRNA lentiviruses according to manufacturer′s instructions. After cultured for 10 days, the surviving colonies ($\geq$50 cells/colony) were counted after staining with 0.2% crystal violet.

LUCIFERASE ASSAYS FOR TOPFLASH REPORTER GENE ACTIVITIES

The luciferase assays were performed according to the manufacturer′s protocol. Briefly, A549 cells were seeded onto 24-well plates with a density of 2×10^5 cells each well the day before transfection. Then, the cells were transfected with 100 ng TOPflash, 1 ng pRL-TK along with psilencer 4.1-empty, psilencer 4.1-NLK-shRNA#1 or psilencer 4.1-NLK-shRNA#2 target constructs using lipofectamine 2,000 reagent according to the manufacture′s protocol. The cells were lysed 48 h after transfection and subject to dual luciferase reporter gene assay. The experiments were repeated for three times.

REAL-TIME PCR ANALYSIS

The total RNA of A549 cells infected with NLK-shRNA and control-shRNA lentiviruses were extracted using a SuperfectRI reagents (Shanghai Pufei Biotechnologies). Then, 1 μg of total RNA was converted to cDNA using the First Strand cDNA Synthesis Kit (Fermentas Scientific, Inc.). Real-time PCR was performed using the LightCycler 480 system with a 96-well plate. The primers used for real-time PCR are as follow: DKK1, 5′-CCGAGGAGAAATTGA-GGAAAC-3′, and 5′-TCTTGGACCAGAAGTGTCTAGC-3′; Axin2, 5′-GGCCAGTGAGTTGGTTGTCAC-3′, and 5′-CCTTCATACATCGG-GAGCACC-3′; β-actin, 5′-AGGCCAACCGCGAGAAGAT-3′, and 5′-TCACCGGAGTCCATCACG-AT-3′.

STATISTICAL ANALYSIS

The SPSS13.0 statistical program was used for statistical analysis. The NLK expression and clinicopathological features were analyzed using the χ^2 test. For analysis of survival data, Kaplan–Meier curves were constructed, and the log-rank test was performed. Multivariate analysis was performed using Cox′s proportional hazards model. The risk ratio and its 95% confidence interval were recorded for each marker. $P < 0.05$ was considered statistically significant for all of the analysis.

DENSITOMETRIC ANALYSES

The density of specific bands were measured by a computer-assisted image analysis system (Adobe Systems, San Jose, CA) and normalized against GAPDH levels. The relative differences between the control and the experimental groups were calculated and expressed as relative increases, which was set control as 1. The results are responsible for at least three independent reactions.

RESULTS

NLK WAS DOWNREGULATED IN LUNG CANCER TISSUES

To explore the clinical significance of NLK in NSCLC progression and further characterize the relationship between NLK and PCNA, we investigated their abundance by Western blot. In eight paired fresh lung cancer and non-tumorous adjacent tissues, the relative abundance of NLK and PCNA appeared to exhibit an inverse correlation. Compared with the non-tumorous adjacent ones, NLK expression dramatically decreased while PCNA upregulated in the tumor tissues (Fig. 1). To confirm the expression of NLK in NSCLC, we investigated the expression of NLK by using IHC on 83 samples from patients with NSCLC. Expectedly, NLK was lowly expressed in poor differentiated specimens compared with well-differentiated ones, which was in inverse correlation with Ki-67. Representative examples of reactivity for NLK and Ki-67 are shown in Figure 2. NLK was expressed mainly in the cytoplasm, whereas Ki-67 was located in the nucleus (Fig. 2). Accordingly, NLK was downregulated in NSCLC specimens, and might be an important protective factor in NSCLC.

CORRELATION OF NLK EXPRESSION WITH CLINICOPATHOLOGICAL FEATURES IN NSCLC

To further explore the relationship between the expression of NLK and Ki-67 in NSCLC, the clinicopathological data are summarized in

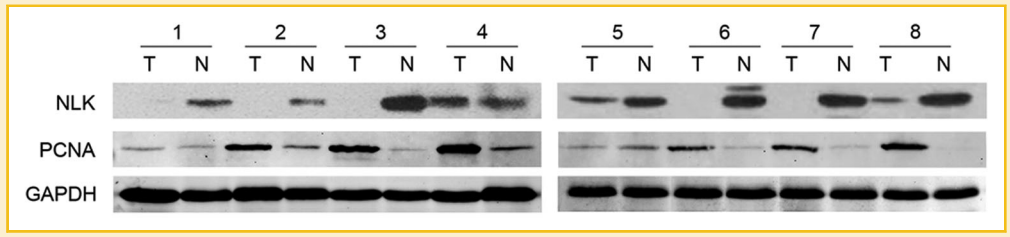

Fig. 1. Expression profiles of NLK in NSCLC and non-tumorous adjacent tissues. Western blot was performed to study the protein levels of NLK in eight representative paired samples of NSCLC tissue (T) and non-tumorous adjacent tissues (N). Compared with non-tumorous adjacent tissues, NLK expression was significantly downregulated while PCNA was obviously upregulated. GAPDH was used as a loading control. The same experiment was repeated at least three times.

Table I. The carcinoma specimens were divided into high expressers and low expressers according to the percentage of Ki-67-positive cells using the cut-off values of 50% representing the mean value of Ki-67 expression, and the average score of 3 for NLK expression, respectively. The results showed that NLK expression was significantly associated with histological differentiation ($P = 0.002$), clinical stage ($P = 0.001$), and lymph node status ($P = 0.015$), but there was no correlation between NLK expression and other prognostic factors such as age, gender, histological type, and tumor size. In addition, a negative correlation between NLK expression and Ki-67-based proliferative activity was found ($P < 0.001$; Table I).

NLK WAS SIGNIFICANTLY ASSOCIATED WITH THE SURVIVAL OF NSCLC PATIENTS

Survival analysis was restricted to 83 patients with follow-up data until death. After all variables were compared separately with survival status, we found NLK ($P = 0.023$), Ki-67 ($P = 0.025$), histologic differentiation ($P < 0.001$), clinical stage ($P < 0.001$), and tumor size ($P = 0.017$) significantly influenced survival (Table II). In univariate analysis, the Kaplan–Meier survival curves showed that high NLK expression correlated with better survival with statistical significance ($P < 0.001$; Fig. 3). In a word, multivariate analysis using the Cox's proportional hazards model proved that NLK ($P = 0.023$; Table II) was an independent prognostic factor for patients' overall survival.

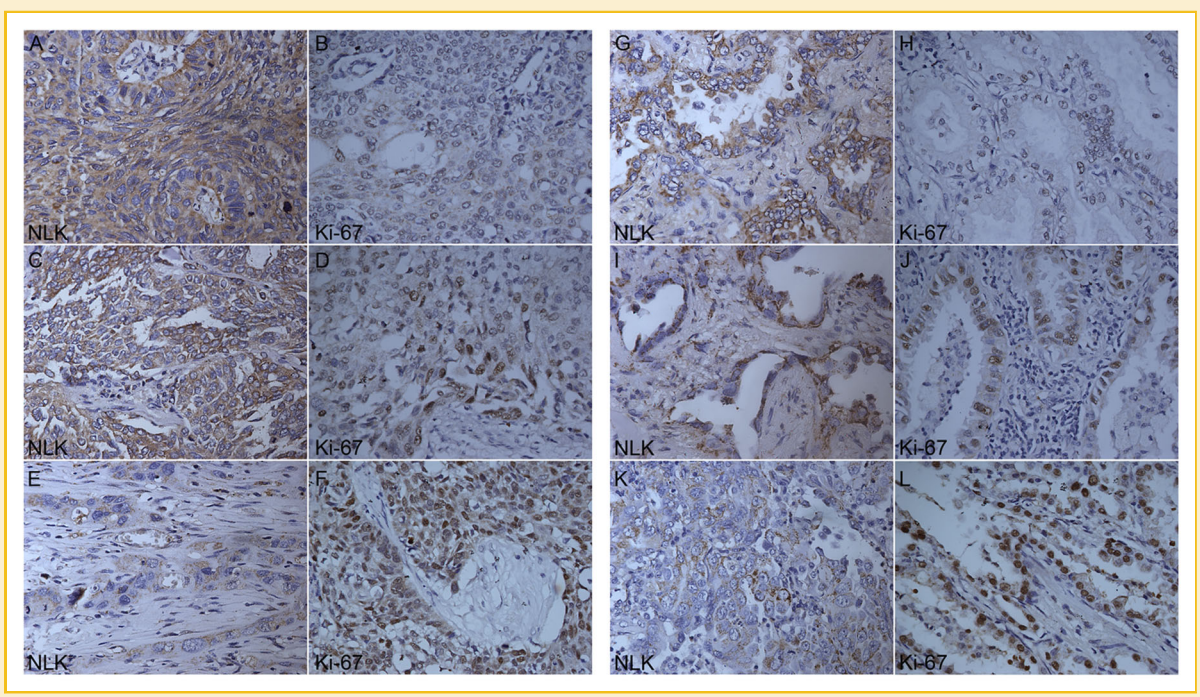

Fig. 2. Representative microphotographs for NLK by IHC in NSCLC. Paraffin-embedded tissue sections were stained with antibodies for NLK and Ki-67 and counterstained with hematoxylin (A–L). NLK was lowly expressed while Ki-67 was highly expressed in lung squamous cell carcinoma cells (A–F) and lung adenocarcinoma cells (G–L). According to the intensity of NLK expression, we divided the samples into histologic differentiation grade 1 (A, B, G, H), histologic differentiation grade 2 (C, D, I, J), and histologic differentiation grade 3 (E, F, K, L).

TABLE II. Contribution of Various Potential Prognostic Factors to Survival by Cox Regression Analysis in 83 NSCLC Specimens

	Hazard ratio	95.0% Confidence interval	*P*
Age	1.018	0.607–1.708	0.945
Gender	0.908	0.462–1.786	0.780
Tumor size	1.907	1.122–3.241	0.017*
Smoking status	1.738	0.864–3.494	0.121
Histological type	0.801	0.548–1.173	0.254
Clinical stage	1.989	1.356–2.917	<0.001*
Histological differentiation	3.416	2.294–5.088	<0.001*
Lymph node status	0.627	0.357–1.100	0.104
NLK expression	0.432	0.210–0.890	0.023*
Ki-67 expression	2.277	1.109–4.676	0.025*

Note: Statistical analyses were performed by the Cox regression analysis.
*$P < 0.05$ was considered significant.

THE ALTERATIONS OF NLK EXPRESSION IN PROLIFERATING A549 CELLS

It was reported that NLK might play a role in cell cycle regulation in various human cancers. We were interested in whether the expression changes of NLK had any influence on cell cycle of NSCLC cells. We next examined whether the expression of NLK was cell-cycle-dependent in A549 cells. As expected, we found that after serum starvation for 72 h, NLK content significantly decreased upon serum addition (Fig. 4A,B). Besides, a concomitant upregulation of PCNA and cyclin A were observed. To confirm the results, flow cytometry analysis was performed to find that the cell population of A549 at G0/G1 phase accumulated after serum starvation. And then upon serum addition, the cells at S phase were increased gradually from 16.26% to 27.99% (Fig. 4C). Thus, these results indicated that the aberrant expression of NLK in NSCLC specimens might have an impact on the proliferation of NSCLC cells.

KNOCKDOWN OF NLK INCREASED THE PROLIFERATION, CLONE FORMATION ABILITY OF A549, AND SK-MES-1 CELLS

To further study the potential effects of NLK on NSCLC cell proliferation, we ablated NLK in both A549 and SK-MES-1 cells using lentivirus-mediated RNA interference approach. Cells were infected with control-shRNA, NLK-shRNA#1, or NLK-shRNA#2 lentiviruses and the expression of NLK was assessed. NLK protein levels were substantially decreased in A549 and SK-MES-1 cells infected with NLK-shRNA lentiviruses compared with the control-shRNA lentiviruses infected cells (Figs. 5A,B and 6A,B). The growth of cells infected with NLK-shRNA lentiviruses was significantly increased, which was consistent with the upregulation of the cell proliferation makers, cyclin A and PCNA (Figs. 5A,B and 6A,B). Furthermore, flow cytometry analyses of cell cycle distribution revealed a decrease of cell number in the G0/G1 phase, with a concomitant augment in the number of cells at S phase from 14.62% to 25.76% and 25.34% compared with control-shRNA (Fig. 5 C),

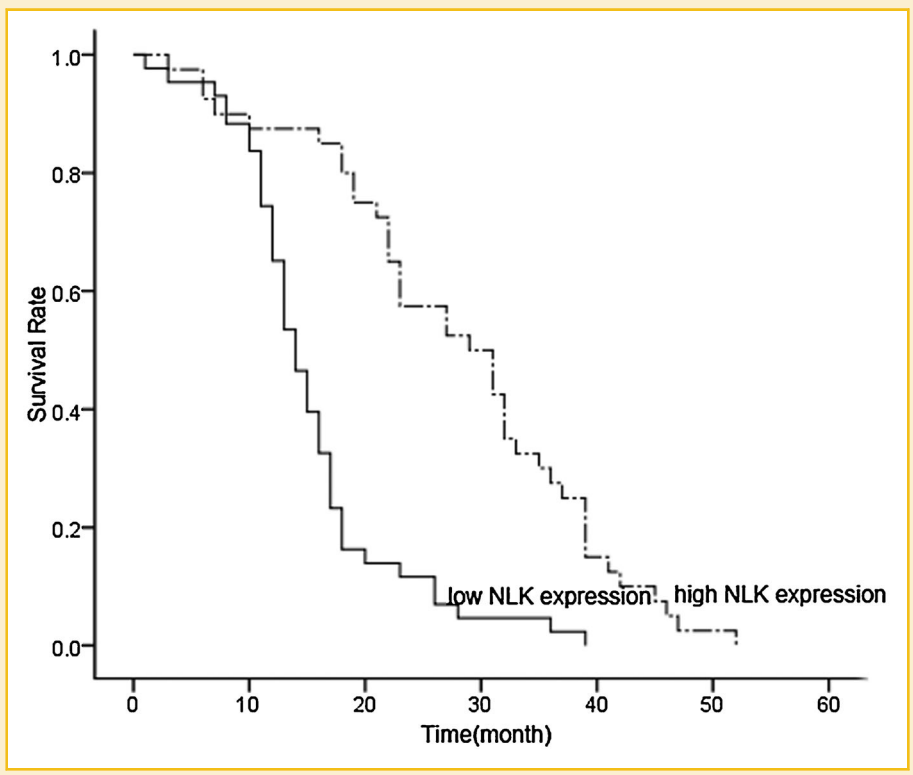

Fig. 3. Correlation between NLK expression and patients' survival. Kaplan–Meier survival curves for low versus high NLK expression on 83 patients with NSCLC showed a highly significant separation between curves ($P < 0.001$).

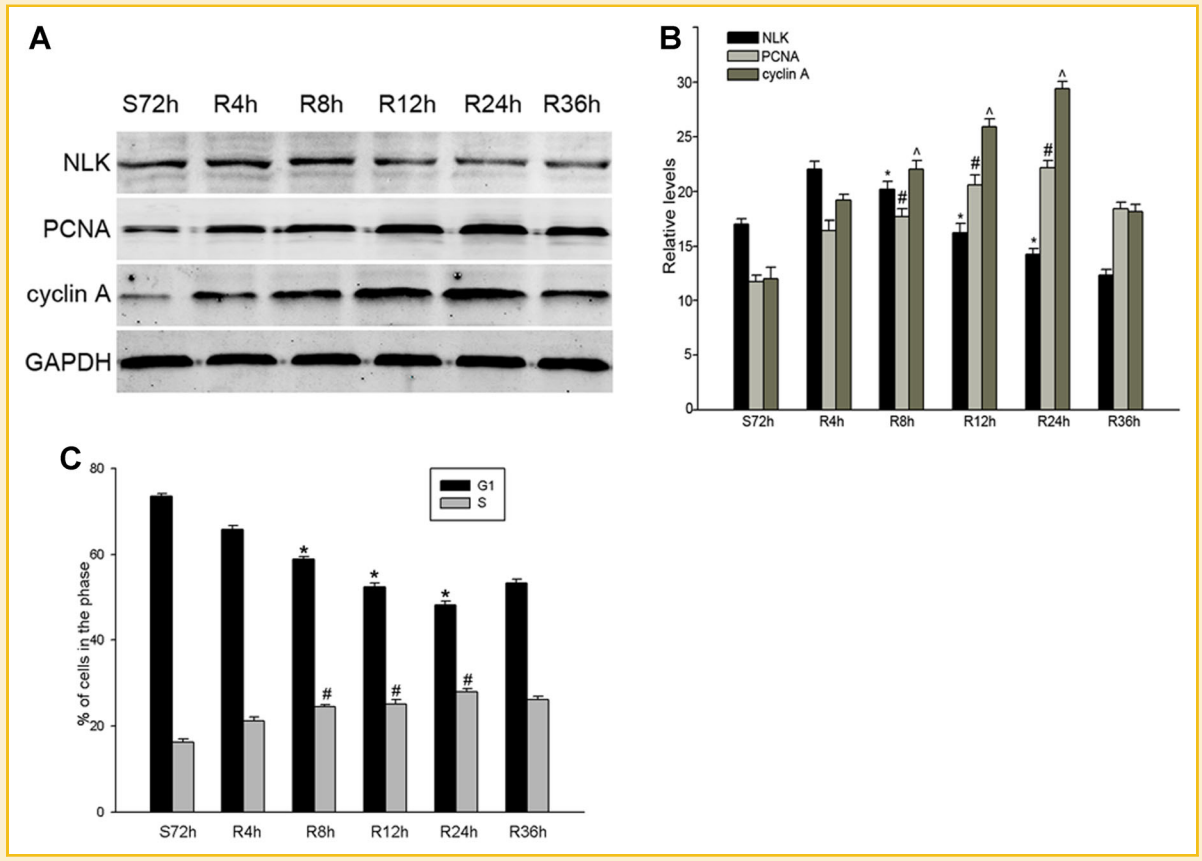

Fig. 4. Expression of NLK and cell-cycle related molecules in proliferating A549 cells. The S72h A549 cells were released by refeeding with serum, and cell lysates were prepared and analyzed by Western blot using antibodies against NLK, cyclin A, PCNA, and GAPDH (A). The bar graph indicates density of NLK/cyclin A/PCNA versus GAPDH at each time point (B). Cells synchronized at G1 progressed into the cell cycle when serum was added for 0, 4, 8, 12, 24, or 36 h (C). Data are presented as means ± SEM of three independent experiments. *, #, ^$P < 0.05$, compared with control cells serum starved for 72 h (S72h). S, serum starvation; R, serum release; SEM, standard error of the mean.

suggesting that NLK could delay G0/G1-S transition of cell cycle, and thus might be responsible for the growth promotion of A549 cells. It was also verified in SK-MES-1 cells (Fig. 6C). To confirm the effect of NLK, we further used CCK-8 to test the proliferation of NSCLC cells. The proliferation was dramatically enhanced in NLK-shRNA, compared with control-shRNA in A549 and SK-MES-1 cells (Figs. 5D and 6D). Besides, using clone formation assay, the formed clones of NLK-shRNA was two times more than the control group (Fig. 5E,F). Taken together, we confirmed that knocking down NLK had an influence in promoting the proliferation of A549 cells.

NLK INHIBITS THE ACTIVITY OF WNT/β-CATENIN SIGNALING IN A549 CELLS

As a negative regulator of Wnt signaling pathway, NLK has been reported to suppress ovarian cancer and glioma proliferation. Consequently, to determine whether NLK had a similar effect on Wnt/β-catenin signaling pathway in NSCLC cells, we analyzed the effect of NLK on β-catenin/TCF transcriptional activity using dual luciferase reporter gene assay, which showed that the activity of β-catenin/TCF was much higher in NLK-depleted A549 cells (Fig. 7A). Furthermore, the knock-down effect of NLK was detected by Western blot, which also showed that the expression of two well-known β-catenin/TCF target genes, c-myc and cyclin D1, both of which were important proteins for cell cycle regulation, while a significant increase was achieved with depletion of NLK in A549 cells (Fig. 7B,C). What is more, Real-time PCR analysis was done to imply that two inhibitors of Wnt signaling pathway like DKK1 and Axin2 were up-expressed when NLK was knocked down (Fig. 7D). These results suggested that NLK was an inhibitor of Wnt signaling pathway in NSCLC, which was possibly responsible for its role in hampering NSCLC development.

DISCUSSION

In this study, we demonstrated that NLK might be an important regulator in NSCLC. Using Western blot and IHC analysis, we found that NLK was downregulated in NSCLC tissues compared with non-tumorous adjacent samples. We also found that the proliferation of NSCLC cells was significantly promoted when NLK was knocked down. Furthermore, using dual luciferase reporter gene and Western blot assays, NLK was found to negatively regulate Wnt/β-catenin signaling in NSCLC cells. Our results indicated that NLK was a negative regulator of NSCLC, probably through the negative regulation of Wnt signaling.

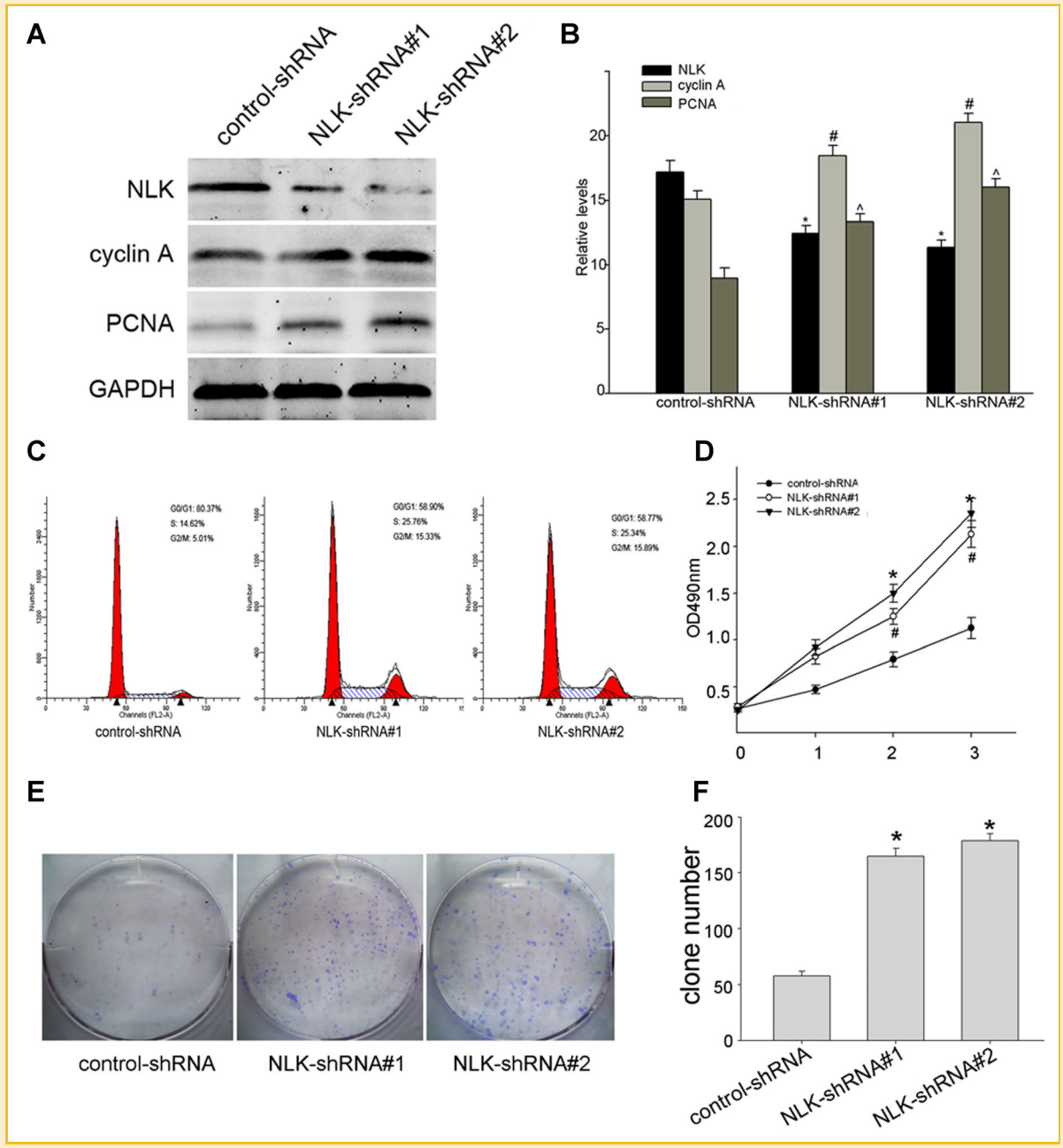

Fig. 5. Inhibition of A549 cell proliferation by knocking down NLK expression. Western blot showed shRNA lentiviruses silenced NLK in A549 cells. Knocking NLK down induced increasing profiles of cyclin A, PCNA in shRNA lentiviruses treated cells (A). The bar chart indicated density of NLK/cyclin A/PCNA versus GAPDH (B). Data are presented as means ± SEM (*, #, ^$P < 0.05$). After infected with NLK-shRNA lentiviruses, the adherent A549 cells revealed a decrease of cell number on G0/G1 phase, with a concomitant augment in the number of cells on S phase by flow cytometry (C). NLK-shRNA-treated A549 cells exhibited significantly enhanced proliferation (D). The data are means ± SEM (*, #$P < 0.05$). Clone formation analysis of shRNA-NLK and shRNA-control treated A549 cells demonstrated the formed clones of NLK-shRNA was two times more than the control group (E, F). Data are presented as means ± SEM (*$P < 0.05$).

Recently, it has been revealed that deregulation of NLK is closely linked to human cancers, such as glioma cancer [Cui et al., 2011] and HCC [Jung et al., 2010], which strengthens the significance of NLK as a promising clinical therapeutic candidate. High expression of NLK suppresses the growth of several cancer cell lines, indicating that this serine/threonine kinase has a critical effect in the regulation of cell growth in cancer. However, the role NLK acting in NSCLC is still unknown. In the present study, we first identified the expression of NLK in 8 paired fresh lung cancer and non-tumorous adjacent tissues (Fig. 1). Interestingly, the expression of NLK in NSCLC was lower than that in non-tumorous adjacent lung tissues. And then, we performed IHC on 83 paraffin-embedded slices. Expectedly, the intense of NLK staining was lightened with the differentiated deteriorated (Fig. 2). Besides, a negative correlation between NLK expression and Ki-67-

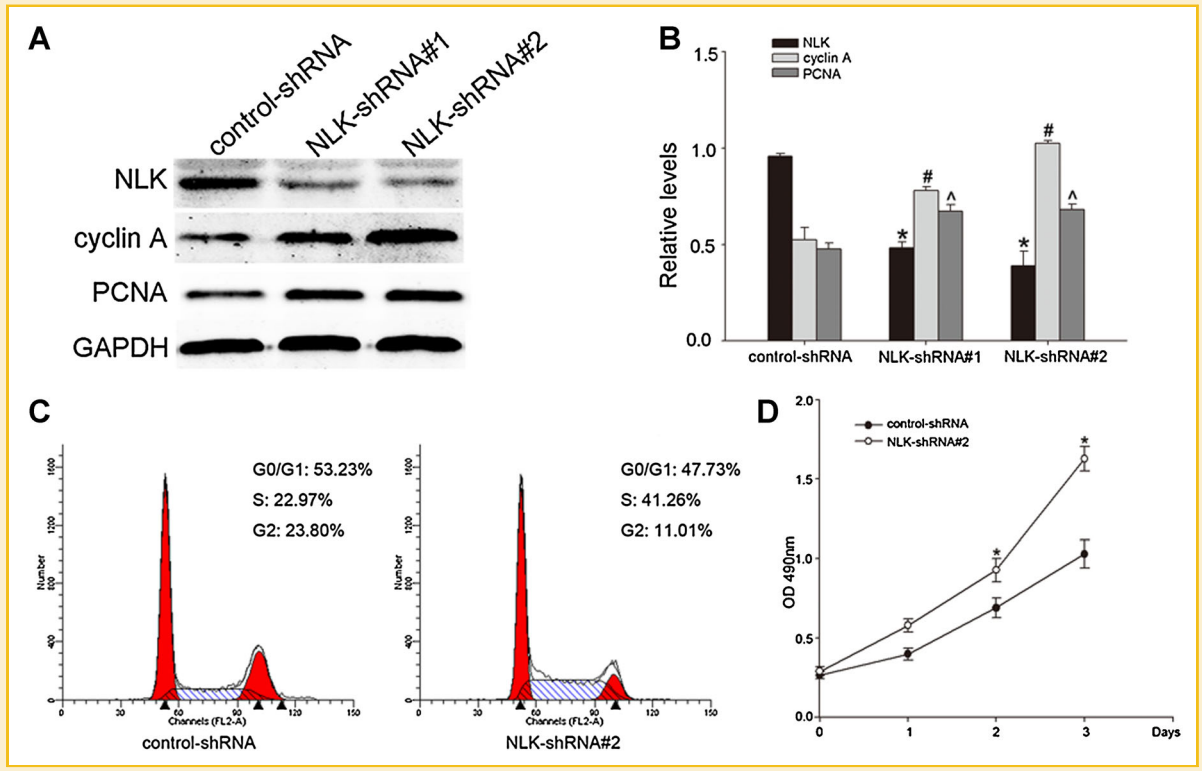

Fig. 6. Suppression of SK-MES-1 cell proliferation by downregulating NLK expression. NLK expression was downregulated, while cyclin A, PCNA were enhanced in cells infected with lentiviruses versus GAPDH (loading control) by Western blot (A). The bar chart below demonstrated the ratio of NLK/cyclin A/PCNA protein to GAPDH by densitometry (B). The data are means ± SEM (*, #, ^$P < 0.05$ compared with the control). Cell cycle analysis by flow cytometry in SK-MES-1 cells with NLK downregulation showed a corresponding augment of cell numbers on S phase (C). Cell proliferation was promoted in NLK-shRNA-treated SK-MES-1 cells using the CCK-8 assay (D). Data are means ± SEM (*$P < 0.05$ compared with the control). The experiments were repeated three times.

based proliferative activity was found ($P < 0.001$; Table I). Oncogenesis is a complex process, low-expression of NLK may represent an important mechanism in the development of NSCLC. In addition, we evaluated the correlation between NLK and clinicopathological features, as well as prognosis. We found that NLK expression was significantly associated with clinical stage ($P = 0.001$), histological differentiation ($P = 0.002$), and lymph node status ($P = 0.015$), but there was no correlation between NLK expression and other prognostical factors (Table I). Besides, high expression of NLK was significantly associated with better prognosis ($P < 0.001$, Fig. 3). These findings indicated that NLK was probably a reliable indicator for prognosis in patients with NSCLC.

As an atypical MAPK member, NLK plays important roles in the regulation of many pathways [Kim et al., 2010], including Wnt/β-catenin, TGF-β, NF-ΚB, and p53 [Avantaggiati et al., 1997], which are of vital importance in oncogenesis. Thus, it is possible that NLK may also participate in regulating cancer progresses. And the following experimental data supported our hypothesis. However, unlike most MAPK members, NLK is more likely to serve as a negative regulator in cancer progression, which shows its unusual property in regulating cancer development. Indeed, NLK is prone to negatively regulate its downstream signals, among which NLK's role in modulating Wnt/β-catenin pathway is the most widely studied [Zanotti and Canalis, 2012]. NLK is an antagonist of the canonical Wnt/β-catenin pathway [Yasuda et al., 2004]. When NLK directly phosphorylates TCF/LEF in the cytoplasm, the combination of TCF/LEF with β-catenin is inhibited, and then the activation of transcriptional complex is abolished [Ishitani et al., 2003]. Consistent with the previous reports, we did find that the transcriptional activity of TCF/LEF was elevated when NLK was knocked down. Thus, the function of NLK in negatively regulating Wnt/β-catenin pathway may account for its role in NSCLC development.

The Wnt signaling pathway regulated cell proliferation and differentiation in various organisms [Katoh, 2007]. β-Catenin is a critical molecular in the Wnt signaling pathway. When accumulating in the nucleus, β-catenin activates the TCF/LEF complex and the transcription of target genes, including cyclin D1, c-myc [Sampson et al., 2001], both of which are important molecules in promoting cell proliferation. What's more, we found the expression of both DKK1 and Axin2 were upregulated in NLK-knocked down cells. The roles of wnt inhibitors in wnt signaling were very intriguing. First, wnt inhibitors were essential for the tight control of wnt signaling. On the other hand, many wnt inhibitors are direct target of LEF/TCF transcription complexes. Thus, LEF/TCF-mediated DKK1 and Axin2 expression would form a feedback loop to prevent the hyperactivation of wnt signaling (Jho et al., 2002; Niida et al., 2004). Recently, it was reported that DKK1 was highly expressed in a variety of cancer types, including lung cancer [Sheng et al., 2009].

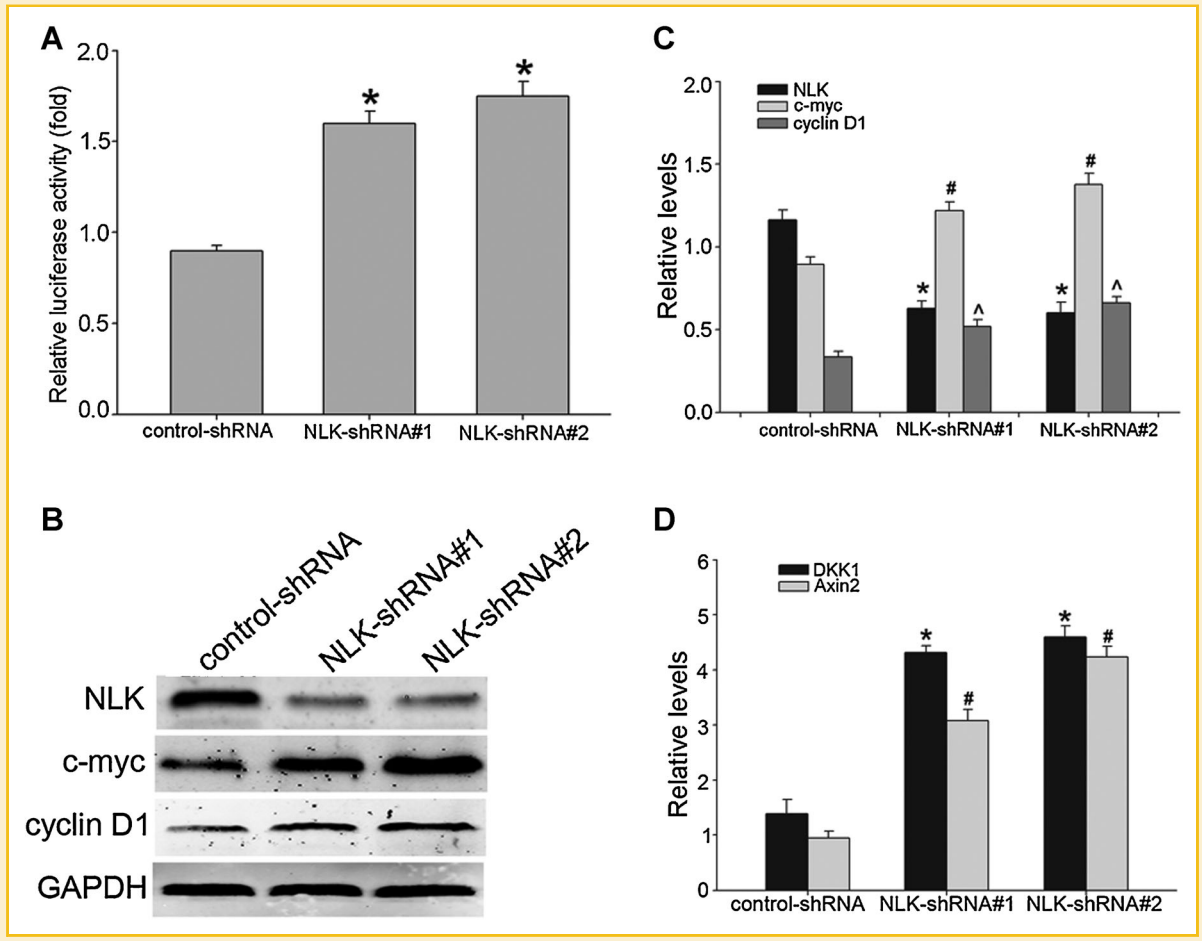

Fig. 7. Antagonization of Wnt/β-catenin signaling pathway by NLK. Knocking down NLK increased the activity of β-catenin/TCF reporter gene, which was measured by dual luciferase reporter gene assay. The activity of TOPFlash in control vector-transfected cells was defined as 1 (A). Data are means ± SEM (*$P < 0.05$). The knockdown of NLK increased the expression of cyclin D1 and c-myc in A549 cells. GAPDH was used as internal reference (B). The bar chart below demonstrated the ratio of c-myc, cyclin D1 protein to GAPDH by densitometry (C). The data are means ± SEM (*, #, ^$P < 0.05$). The knockdown of NLK upregulated the expression of DKK1, Axin2 in A549 cells by Real-time PCR analysis. β-Actin was used as an internal reference. The bar chart below demonstrated the ratio of DKK1, Axin2 RNA levels to β-actin by densitometry (D). The data are means ± SEM (*, #$P < 0.05$). All the results were repeated at least three times.

Importantly, it was suggested that the role of DKK1 as wnt inhibitor could be inactivated in cancer cells, implying that the disruption of the feedback mechanism would be a critical event for uncontrolled wnt signaling in cancer cells. Therefore, we speculated that the upregulation of DKK1 and Axin2 in NLK knockdown cells was an important feature of hyperactivation of wnt signaling. Thus, hyperactivated Wnt/β-catenin signaling would result in uncontrolled cell growth and -cause cancers. Dysregulation of the Wnt signaling pathway is involved in many malignancies, including colon [Rosenbluh et al., 2012] and lung cancers [Nguyen et al., 2009]. Posttranscriptional modification of the components of β-catenin and TCF/LEF complex that abolishes constitutive activation of the Wnt signaling pathway may prevent cancer progression. Although the dysregulation of Wnt signaling pathway in various types of cancers has been implied, such mechanism is poorly investigated in NSCLC. In our study, we confirmed that NLK was a regulator of Wnt signaling and affected its downstream proliferation-regulating genes in NSCLC. Thus, the decreased expression of NLK in NSCLC may contribute to hyperactivated Wnt/β-catenin activity, which in turn promotes cell proliferation.

In conclusion, NLK, as a negative transcriptional regulator of Wnt/β-catenin signaling pathway, may contribute to the progression of NSCLC based on our observations. NLK was decreased in NSCLC and negatively correlated with NSCLC cell proliferation. All the studies indicated that upregulated NLK levels may be a promising target for the chemotherapy. Hence the regulation of NLK/Wnt/β-catenin signaling pathway is likely to become a useful tool for both diagnostic and prognostic applications in NSCLC.

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