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Knockdown of NF-κB p65 subunit expression suppresses growth of nude mouse lung tumor cell xenografts by activation of Bax apoptotic pathway

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Nuclear factor-kappaB (NF-κB) is an important transcriptional factor and regulates a variety of pathophysiologic process involved in cell survival and death. The present study assesses the effects of NF-κB p65 subunit knockdown in suppression of nude mouse lung tumor cell xenografts and understands the underlying molecular events.

A nude mouse Lewis lung carcinoma cell xenograft model was established and the mice were intraperitoneally injected with NF-κB p65 siRNA and sacrificed after two weeks of tumor cell injection. Tumor xenografts were harvested for TUNEL, Western blot, and qRT-PCR analyses.

Compared to the PBS-treated or the negative control (NC) siRNA-treated mice, **t**umor xenograft weight and volume was significantly decreased in the NF-κB p65 siRNA-treated mice. The TUNEL positive (apoptosis) cells in xenograft sections were 45 ± 5 in PBS and 38 ± 3 in NC siRNA, but increased to 271 ± 11 in p65 siRNA-treated mice. Compared to the PBS or the NC mice, levels of Bax mRNA and protein in tumor xenografts were significantly upregulated in p65 siRNA-treated mice.

Knockdown of NF-κB p65 subunit expression significantly inhibited the growth of nude mouse Lewis tumor cell xenografts by induction of tumor cell apoptosis and significantly up-regulation of pro-apoptotic protein Bax expression. Future study will confirm the current data and targeting NF-κB p65 subunit expression as a potential therapeutic strategy in treating human lung cancer.

Key words: NF-κB, lung cancer, apoptosis, small interfering RNA, xenografts

Lung cancer is the most common malignant tumor with an incidence of 12.8% rate among all cancers with the leading cause of cancer-related deaths in the world [1, 2]. With the increase in air pollution in the developing countries, lung cancer incidence rate is increasing [3]. Most lung cancers are diagnosed at the advanced stage of disease, making curable surgery impossible. Chemo- and radiotherapy is currently the major therapeutic option in management of advanced inoperable lung cancer patients in clinical practice [4], although chemotherapy often generates the drug resistance and toxicity [5]. Lung cancer prognosis is generally poor and to date only approximately 15% patients survive for five years after lung cancer diagnosis. Therefore, there is an urgent need on effective prevention and developing effective therapy to reduce the morbidity and mortality of lung cancer.

Cancer development and progression rely on the balance between tumor cell survival and apoptosis [6, 7]. Nuclear factor-kappaB (NF-κB) is an important transcriptional factor in regulation of a variety of pathophysiologic processes involved in cell survival and death, especially during carcinogenesis and cancer progression [8-11] by inhibiting the apoptosis thereby enhancing the tumor growth [12, 13]. Level and activity of NF-κB protein have been found constitutively increased in a variety of human cancers [12, 13]. Recent *in vivo* and *in vitro* studies suggested that NF-κB was closely associated with lung carcinogenesis [14-16]. Studies of some lung cancer cell lines have suggested that different drugs (such as Querceti and Plumbagin) induced apoptosis was mediated by inactivation of NF-κB pathway [17-20]. However, the similar results in lung cancer have not been reported.

Thus, targeting of the NF-κB signaling pathway has significant implication in treatment and chemoprevention of lung cancer [21-23]. For example, inhibition of NF-κB activity is associated with increased sensitivity of lung cancer cells to chemotherapeutic agents [24]. NF-κB protein consisted of a DNA binding subunit p50 and a transactivation subunit

RelA/p65. These two subunits form a heterodimer to function as a transcriptional factor, i.e., when it is not activated, NF-κB dimer is localized in the cytoplasm as a latent complex by binding specifically to IκB inhibitor proteins. After being activated, IκB inhibitor proteins are phosphorylated and subsequently degraded, leading to NF-κB translocation into the nucleus and activates the transcription of NF-κB downstream genes which are related to cell proliferation, survival and anti-apoptosis [14]. In the present study, we utilized siRNA duplex to knockdown expression of NF-κB p65 subunit to assess whether knockdown of NF-κB can inhibit growth of lung cancer cells *in vitro* and *in vivo* nude mouse xenografts and understand the underlying molecular events. This study expects to provide evidence whether targeting the NF-κB activity could be an effective therapeutic strategy in future clinical control of lung cancer.

Materials and methods

Cell line and culture. A Lewis murine lung carcinoma cell line was obtained from Jin Zijing Company (Beijing, China) and maintained in RPMI1640 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Hyclone, Beijing, China), 100 U / mL penicillin, and 100 pg / mL streptomycin in a humidified incubator with 5% CO_2 at 37°C.

In vivo tumorigenicity. To assess the effects of NF-κB p65 subunit knockdown, we performed a nude mouse xenograft assay by using 18 healthy male C57BL nude mice (age of 6-8 weeks and body weight of 20±2 g, maintained in the animal facility at China Medical University). The care and use of laboratory animals was in accordance with the principles and standards set forth in the Principles for Use of Animals at China Medical University. All animal procedures were approved by the Institutional Animal Care and Use Committee. In brief, the Lewis lung carcinoma cells were grown to 75% confluence, trypsinized, washed twice in phosphate buffered saline (PBS), and resuspended in the growth medium. Next, 0.5 ml cell suspension at a density of 2×10^7/mL was subcutaneously injected into forelimbs of each of three nude mice. Two weeks after tumor cell inoculation, three mice were sacrificed and the subcutaneous tumors were removed for tumor cell suspension. After that, 0.2 ml of tumor cell suspension at a density of 6×10^6 cells was subcutaneously injected into forelimbs of each of 15 mice. The mice were randomly divided into different treatment groups, i.e., i). PBS control group, intraperitoneal injection of PBS; ii). siRNA negative control group, intraperitoneal injection of negative control siRNA with the final concentration of 100 nmol / L; and iii). p65 siRNA group, intraperitoneal injection of p65 siRNA with the final concentration of 100 nmol / L. p65 siRNA sequence was 5'-GATCAATGGCTACACAGGA-3' and negative control siRNA sequence was 5'-TTCTCCGAACGTGTCACGT-3' (Both were purchased from Santa Cruz biotechnology, Santa Cruz, CA, USA).

The tumor volume and inhibition of tumor growth were recorded every other day from the beginning of the siRNA treatment. The nude mice were weighted and tumor dimension was measured with calipers. The tumor volume was calculated by using the formula: Volume (mm^3) =1 / $2AB^2$. A represents the long diameter (mm) and B represents the short diameter (mm). After two weeks following the treatment, the mice were sacrificed and the tumor mass was resected and weighed. Tumor weigh and inhibition rate were calculated and plotted using the formula: tumor inhibitive rate = [(the average tumor weight of control group - the average tumor weight of siRNA group) / the average tumor weight of control group] × 100%.

***In situ* TUNEL apoptosis assay**. To determine whether inhibition of NF-κB by siRNA induces tumor cells to undergo apoptosis, we measured apoptosis levels in tumor xenograft tissue specimens by using the TUNEL detection kit (Tiangen, Beijing, China). In particular, formalin-fixed, paraffin-embedded tumor xenografts were cut into 4-mm-thick sections. After deparaffinized in xylene and rehydrated through graded ethanol, the tissue sections were then stained according to the kit protocols. At the end, apoptotic cells from randomly selected five visual fields (X 200) in each slide were counted under a double-headed light microscope by two investigators who didn't know about the identity of the slides, which obtained approximately 500 TUNEL positive cells in each slide (Cells with brown granules in the nucleus were TUNEL positive cells).

Quantitative RT-PCR. Total cellular RNA was isolated from tumor xenograft tissue sections using a Trizol (Tiangen) and reversely transcribed into cDNA using an RT kit (Tiangen) according to the manufacturers' instructions. qPCR was performed in triplicates, i.e., the reaction system includes 1 μL cDNA template, 0.5 μL of 10 μM primers, 10 μ L SYBR GREEN master mix, up to 20 μL ddH_2O. The reaction conditions were 95°C for 10 min and then 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 30 s and stored at 4°C. An Exicycler™ fluorescence quantitative analysis system (BIONEER, Korea) was used. All primers were synthesized in SANGON Biological Engineering (Shanghai) Co., Ltd. (Shanghai, China). The DNA sequences of primers were listed in Table 1.

Protein extraction and Western blot. Total cellular protein was extracted from 20 mg tumor xenograft tissue using 100 μL protein lysis buffer containing 50 mM Tris (pH 7.4),

Table 1. Oligonucleotide primers for real-time PCR

Gene	Sequences	Size (bp)
NF- κ B/ P65	Forward: 5'-AGCATTAACCTCCTGGAGACG-3'	224
	Reverse: 5'-TTGGGAGCACTGCTTTGGAT-3'	
Bax	Forward: 5'-CCAGGATGCGTCCACCAAGAA-3'	199
	Reverse: 5'-AGCAAAGTAGAAGAGGGCAACCAC-3'	
β-actin	Forward: 5'-CTGTGCCCATCTACGAGGGCTAT-3'	155
	Reverse: 5'-TTTGATGTCACGCACGATTTCC-3'	

150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA, and leupeptin. 40 μg of protein sample was resolved in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA). After that, the membrane was blocked in 5% skimmed milk for 2 h, and blotted with an anti-p65 antibody at a dilution of 1:400 or anti-Bax antibody at a dilution of 1:1000) (all from Santa Cruz biotechnology) at 4°C overnight. The membrane was washed with PBS-T and incubated with a second antibody at room temperature for 1 h. After washing in PBS-T, the protein bands were visualized using an ECL kit (Biyuntian, Shanghai, China) and exposed to x-ray film (Fujifilm, Tokyo, Japan) and quantified using Gene Tools software (Media Cybernetics, MD, USA).

Statistical analysis. *SPSS* version 13 software (SPSS, Chicago, IL, USA) was used for statistical analysis. Each experiment was repeated three times and the results were expressed as Mean ± SD. P<0.05 was considered as statistically significant.

Results

Knockdown of NF-κB p65 expression inhibits growth of Lewis lung carcinoma cell xenograft in nude mice. To evaluate the effect of NF-κB p65 knockdown on inhibition of growth of nude mouse lung cancer cell xenografts, we first established this animal model and treated these mice with p65 siRNA and controlled with PBS and negative control (NC) siRNA treatment. After two weeks, the animals were sacrificed and the data showed that p65 siRNA treatment significant suppressed growth of tumor xenograft and volume and weight were compared to either the PBS treated or NC siRNA treated mice (Figure 1A and 1B). The inhibition rate of tumor growth was nearly 30-fold more than that in the PBS group (*P<0.05; Figure 1C).

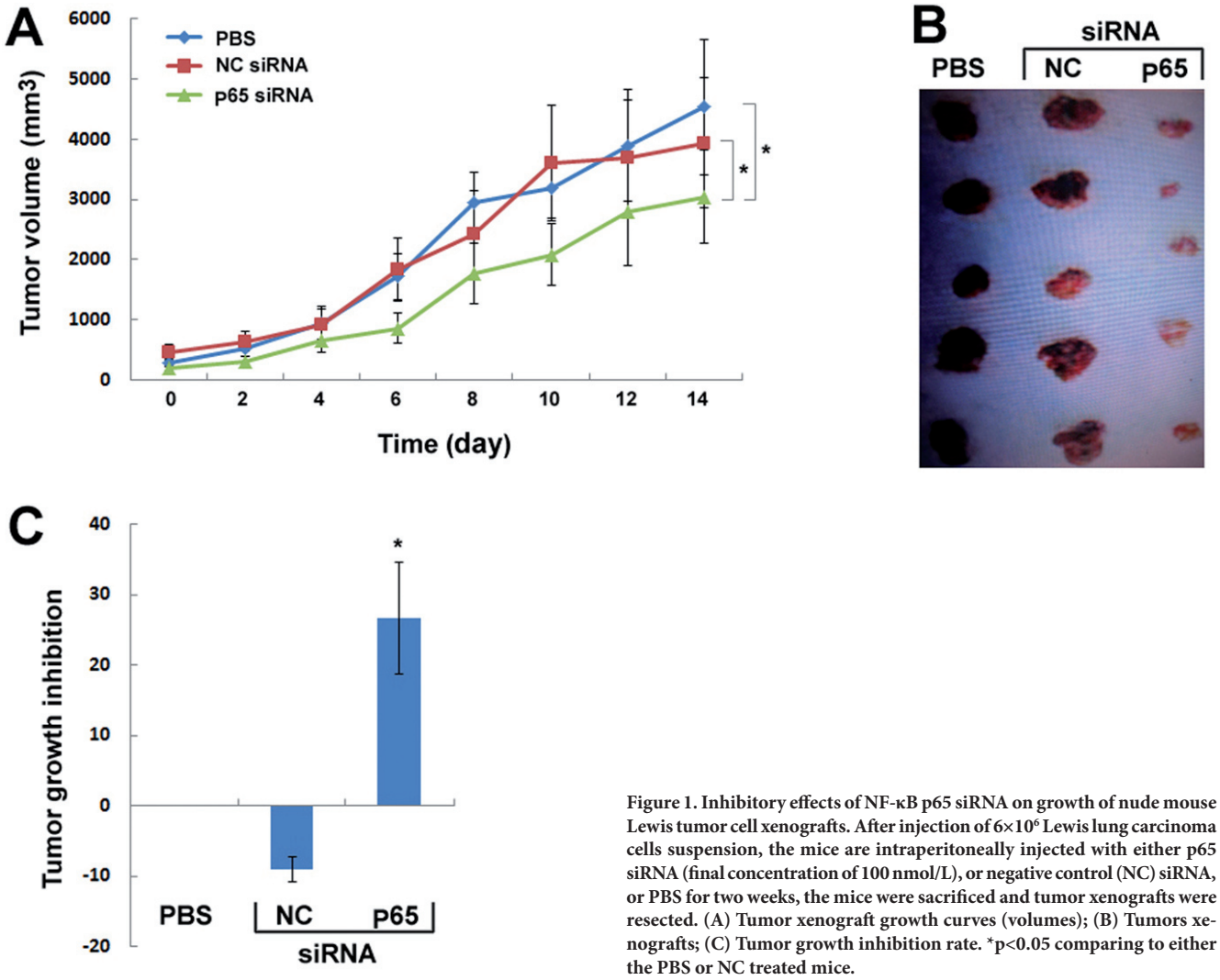

Figure 1. Inhibitory effects of NF-κB p65 siRNA on growth of nude mouse Lewis tumor cell xenografts. After injection of 6×10^6 Lewis lung carcinoma cells suspension, the mice are intraperitoneally injected with either p65 siRNA (final concentration of 100 nmol/L), or negative control (NC) siRNA, or PBS for two weeks, the mice were sacrificed and tumor xenografts were resected. (A) Tumor xenograft growth curves (volumes); (B) Tumors xenografts; (C) Tumor growth inhibition rate. *p<0.05 comparing to either the PBS or NC treated mice.

Knockdown of NF-κB p65 expression induces apoptosis of tumor xenografts. The TUNEL assay data showed that apoptotic cells in tumor xenograft sections were 45 ± 5 in PBS-treated mice and 38 ± 3 in NC siRNA-treated mice, but increased to 271 ± 11 in p65 siRNA-treated mice ($p<0.05$; Figure 2D). NF-κB p65 siRNA significantly inhibited p65 expression ($P<0.01$; Figure 3) but induced the increase in levels of Bax mRNA and protein, an important pro-apoptotic protein compared to the controls ($P<0.01$; Figure 3).

Discussion

Small interfering RNA (siRNA) is an established and powerful technique to knockdown gene expression by post-transcriptional regulation of gene translation or mRNA half life [15]. To date, RNA interference is frequently used in *in vitro* and *in vivo* to inhibit gene expression and assess functions of the target genes or evaluate therapeutic strategies of certain gene silencing. NF-κB is a key transcriptional factor, which has important anti-apoptotic properties and has been linked to cancer cell growth and proliferation in *in vivo* and *in vitro* conditions [11, 16, 25-27]. To date, accumulating evidence has suggested that NF-κB signaling pathway plays a critical role in carcinogenesis and tumor progression by protection from apoptosis and chemoresistance in different cancers [28, 29]. Thus, induction of apoptosis could be an important approach in cancer prevention and treatment. In the present study, we showed that NF-κB p65 siRNA effectively inhibited p65 expression in *in vivo* condition and suppressed growth of nude mouse Lewis lung adenocarcinoma cell xenografts. Our results are consistent with previous studies [30] and further confirmed the role of NF-κB in lung carcinogenesis and progression. Targeting of NF-κB could have significant implications for treatment of lung cancer in future.

Indeed, NF-κB has been reported to possess a strong anti-apoptotic activity in many tumor cells [31]. Induction of apoptosis may be one of the possible mechanisms by which NF-κB siRNA inhibited growth of lung tumor cell xenografts in the current study. Generation of a large amount of fragmented DNA in tumor cells is one of the most important features of apoptosis [32]. Thus, in our current study, we performed the TUNEL assay to detect apoptotic tumor cells in xenograft tissue sections and found that p65 siRNA treatment induced significant increase of apoptosis in tumor cells. This is consistent with previous studies, which showed that antisense RNA-targeting p65 expression inhibited tumor cell

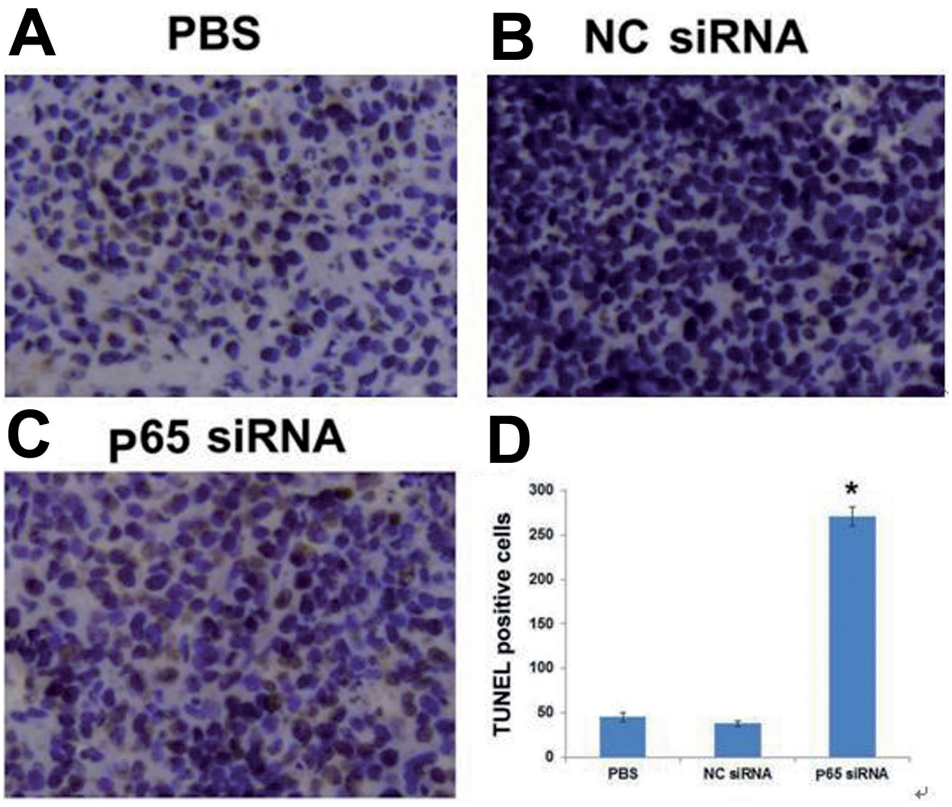

Figure 2. Effects of NF-κB p65 siRNA on induction of Lewis tumor cells to apoptosis assessed by the TUNEL assay. Formalin-fixed, and paraffin-embedded tumor xenografts were sectioned and stained with the TUNEL detection kit. (A) Tumor xenograft sections from PBS-treated mice; (B) Tumor xenograft sections from Negative control siRNA treated mice; (C) Tumor xenograft sections from p65 siRNA treated mice; (D) Summarized data for A to C. *p<0.05 comparing to either the PBS or NC treated group.

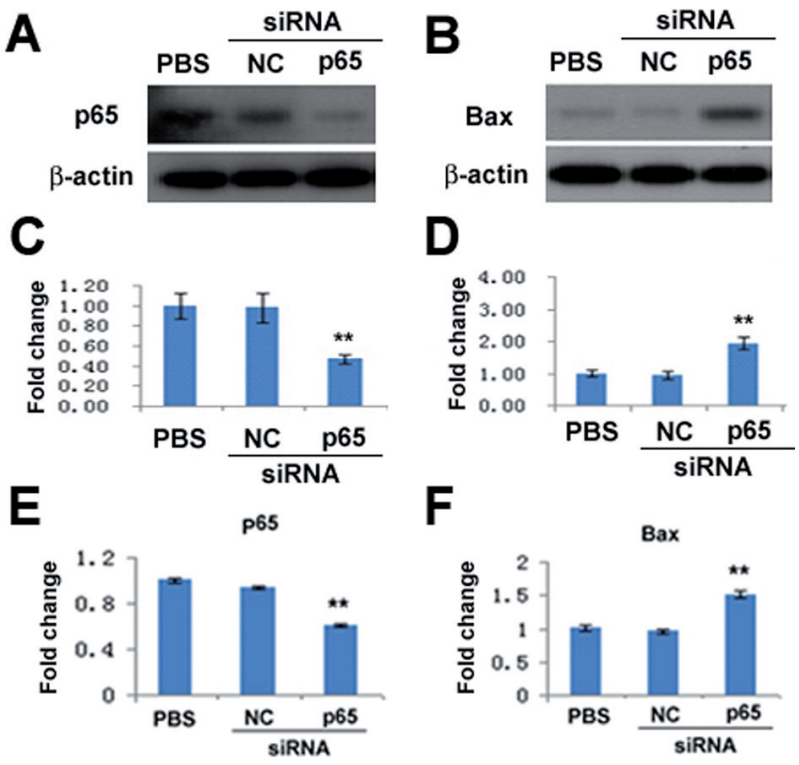

Figure 3. Effects of NF-κB p65 siRNA on regulation of p65 and Bax expression in nude mouse Lewis tumor cell xenografts. (A and B) Western blots of p65 and Bax protein; (C and D) Gel densities of p65 and Bax protein; (E and F) qRT-PCR analysis of p65 and Bax mRNA. **p<0.01 comparing to either the PBS or NC treated group.

growth both *in vitro* and *in vivo* condition [33, 34]. A previous study also demonstrated that inhibition of NF-κB activity using adenovirus mediated IκBa gene transfer enhanced the effects of other anticancer drugs and reduced the dose of chemotherapy agent [35].

Furthermore, our current data showed that knockdown of NF-κB p65 subunit expression was able to significantly up-regulate the Bax expression in tumor xenograft tissue at both protein and mRNA levels. Bax is a pro-apoptotic Bcl-2-family protein that resides in the cytosol and can translocate into the mitochondria upon stimuli and cell apoptosis [36-38]. Bax regulates the critical balance between cellular proliferation and death. Release of cytochrome *c* and other apoptosis-related proteins into the cytosol is a critical process during apoptosis. Previous studies demonstrated that Bax plays an essential role in mediating the mitochondria-dependent apoptosis pathway [39, 40]. Recently, Bax has been shown to induce cytochrome *c* release and caspase activation *in vivo* [41] and *in vitro* [42].

The current study suggests that altered expression of both NF-κB and Bax proteins are closely associated with development and progress of Lewis lung cancer xenograft in C57BL/6 mice. They have an inverse association, i.e., high Bax expression leads to low expression of NF-κB protein and vise verse. Thus, we postulate that during formation and growth of Lewis lung cancer xenograft in C57BL/6 mice, NF-κB signaling pathway was initially activated and inhibits the downstream apoptotic gene Bax expression; thus, in turn reduce tumor cell apoptosis. However, further studies with more cell lines or in vivo experiments are needed to confirm our current data.

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