

Silencing *LMNB1* Contributes to the Suppression of Lung Adenocarcinoma Development

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Purpose: Lung cancer has been recognized as the most fatal malignant tumor with the highest morbidity and mortality in recent years.

Materials and Methods: In this study, we found that *LMNB1*, which is an important component protein of the nuclear skeleton, was significantly upregulated in lung adenocarcinoma (LUAD) and correlated with the pathological stage as well as lymphatic metastasis.

Results: In vitro loss-of-function study utilizing *LMNB1* knockdown LUAD cell lines demonstrated that depletion of *LMNB1* inhibited development of LUAD through regulating cell proliferation, cell apoptosis, cell cycle and cell motility. Decreased tumorigenesis of *LMNB1* knockdown LUAD cells was proved in mice xenograft models. Moreover, the mechanism by which *LMNB1* promotes LUAD was explored through the expression evaluation of apoptosis-related proteins and cancer-related signaling pathways.

Conclusion: In conclusion, our study identified *LMNB1* as a tumor promotor and a potential therapeutic target in LUAD.

Keywords: lung cancer, lung adenocarcinoma, *LMNB1*, cell proliferation, cell apoptosis

Introduction

Lung cancer has been recognized as the malignant tumor with the highest morbidity and mortality in recent years, and the number of morbidities and lung cancer-related deaths keep increasing every year.^{1,2} Non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC) are two main types of lung cancer, of which NSCLC accounts for about 80–85% of all lung cancer cases.³ Moreover, lung adenocarcinoma (LUAD) is the most common subtype of NSCLC.⁴ Although considerable progress has been made in early screening of lung cancer and minimally invasive surgery in recent years, the five-year survival rate of NSCLC is still relatively low. At the beginning of this century, as gefitinib was put into clinical use, the door to molecular targeted therapy for NSCLC was opened.^{5,6} Nowadays, several molecular targets such as PD-L1, ROS1 and ALK have been discovered and applied for the development of targeted therapy.^{7,8} Therefore, an in-depth understanding of the molecular mechanisms of the occurrence and development of LUAD will help develop more effective targeted therapies to extend the survival time of LUAD patients.

The nuclear fiber layer, mainly a protein network located in the inner layer of the nuclear membrane, can maintain the physiological balance of cells under normal circumstances.⁹ Lamins are classified into type A and type B according to their biochemical properties. Type B lamins includes Lamin B1 (*LMNB1*), Lamin B2

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and Lamin B3, where Lamin B1 is encoded by the *LMNB1* gene. *LMNB1* is an important component protein of the nuclear skeleton.¹⁰ Although the research on the lamin family has focused on type A for a long time, more and more attention has been paid to the function of B-type lamins, especially *LMNB1*, in recent years.¹¹ It has been revealed that, in addition to maintaining the shape and integrity of the nuclear, LMNB1 also plays an important role in the regulation of cell proliferation and senescence, chromosome distribution and aggregation, DNA replication and gene expression, and DNA damage repair.¹² The abnormal expression of *LMNB1* has been proved to be related to nervous system diseases and tumor development.^{13,14} Therefore, the in-depth study of *LMNB1* will lay a foundation for the study of the pathological mechanism of various diseases, and the exploration of new markers and therapeutic targets for tumor treatment.

In this study, through the screening by RNA-seq, we found that *LMNB1* was significantly upregulated in LUAD and associated with the pathological stage as well as lymphatic metastasis. Loss-of-function study revealed the inhibitory effects of *LMNB1* on LUAD development by regulating cell proliferation, apoptosis, migration and cell cycle arrest. In addition, the influence of tumor growth by *LMNB1* was confirmed in vivo. Therefore, the results of this study identified *LMNB1* as a tumor promotor in the development and progression of LUAD, which turns out to be a promising therapeutic target in LUAD treatment.

Materials and Methods

Cell Lines and Transfection

Lung cancer cell lines NCI-H1299 and SPC-A-1 were purchased from Cell Resource Center, Institute of Basic Medicine, Chinese Academy of Medical Sciences, EBC-1 and A549 were obtained from Bena Technology (Hangzhou). EBC-1, NCI-H1299 and SPC-A-1 were cultured in RPMI-1640 medium supplemented with 10% FBS and A549 grow in McCoy's 5A medium supplemented with 10% FBS at 37°C, 5% CO₂ with culture medium changed every three days.

A549 and NCI-H1299 cells were transfected with sh*LMNB1*-plasmid vectors (BR-V108-sh*LMNB1*) using lipofectamine 2000 (Invitrogen). shRNA sequences of *LMNB1* (designed by Shanghai Biosciences Co., Ltd) showed:

5'-CAGCAACAGCTGAATGACTAT-3' (Pbr20463),

5'-TTGGAGACACATCAGTCAGTT-3' (Pbr20464),
5'-ATCCAATAGAAGCTGTGCAAT-3' (Pbr20465).

qRT-PCR

Total RNA from transfected A549 and NCI-H1299 cells was isolated using TRIzol reagent (Thermo Fisher Scientific) and the purity and integrity was assessed by Nanodrop 2000/2000C spectrophotometry (Thermo Fisher Scientific). Total RNA (1 µg) was reversely transcribed to high-quality cDNA with HiScript Q RT qPCR SuperMix kit (Vazyme). qPCR was performed using AceQ qPCR SYBR Green Master Mix (Vazyme). All data were normalized using *GAPDH* and 2^{-ΔΔCt} method were used.

Western Blotting (WB) and Human Apoptosis Antibody Array

Total proteins were extracted with ice-cold RIPA lysis buffer and the concentration were determined by BCA protein reagent kit (HyClone-Pierce). WB assays were performed with equal amounts of purified proteins. Proteins were separated by 10% SDS-PAGE and transferred, then the membranes were immunoblotting with primary antibodies against *LMNB1* (Abcam, Cat. #ab229025), Akt (CST, Cat. #4685), P-Akt (R&D, Cat. #AF887-sp), *CCND1* (cyclin D1, CST, Cat. #2978), *CDK6* (Abcam, Cat. #ab151247), *PIK3CA* (Abcam, Cat. #ab40776) and *GAPDH* (Bioworld, Cat. #AP0063). Goat anti-rabbit (Beyotime, Cat. #A0208) was used as the secondary antibody. Blots were detected using ECL-PLUS/Kit (Amersham).

Proteins were isolated and prepared as WB assay. Human apoptosis antibody array (Abcam, Cat. #ab134001) were used and biotin-conjugated anti-cytokines and HRP-conjugated streptavidin and chemiluminescent detection reagents were added. Spots on the array membrane were detected by ECL-PLUS/Kit (Amersham) and pictured using a camera for imaging.

Celigo Cell Counting Assay

Transfected A549 and NCI-H1299 cells and negative control cells were seeded on a 96-well plate in triplicate for culturing five days. The plate was continuously detected by Celigo (Nexcelom) and the cell proliferation rate was analyzed.

Cell Apoptosis and Cell Cycle

2.5×10³ cells/mL A549 and NCI-H1299 cells were seeded in 96-well plates in triplicate and cultured for five days. For cell apoptosis assay, 10 µL annexin V-APC was added

for staining. For cell cycle assay, cells were stained with cell staining solution (40×PI, 2 mg/mL; 100×RNase, 10 mg/mL; 1×PBS=25:10:1000). The percentage of cell phases was measured using FACScan (Becton Dickinson) to assess the apoptotic rate and cell cycle rate.

Wound-healing Assay

A549 and NCI-H1299 cells were seeded into a 96-well dish and grew for 72 h. Wounds were made with a 96-wounding replicator (VP scientific) and cultured for another 36 h. Photographs of wounds were taken by fluorescence micrograph at eight and 36 h and the migration rate was calculated.

Transwell Assay

A549 and NCI-H1299 cell suspension was seeded on the upper chamber of a 24-well transwell migration insert (Corning). The lower chamber was filled with 600 µL culture medium containing 30% FBS for incubation. The non-migration cells in the upper chamber were removed and the cells adhering to the membrane were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet. Transfer rates were calculated via ImageJ software.

RNA Sequencing

Total RNA of 10 lung cancer tissues and para-normal tissues were isolated by Trizol and the concentration were quantified using a NanoDrop 2000 (Thermo Fisher Scientific) and Agilent 2100 Bioanalyzer RNA Nano Chip (Agilent Technologies). Human GeneChip PrimeView (Affymetrix) was used for microarray processing to determine gene expression according to the manufacturer's instructions. RNA sequencing data processing and analysis were performed with R studio. Canonical pathways, diseases and functions, molecular and cellular processes that are significantly associated with differentially expressed genes (DEGs) in the data sets were determined using Ingenuity Pathway Analysis (IPA) software.

Immunohistochemistry (IHC) and Ki-67 Immunostaining

Lung cancer and paranormal tissue microarray was obtained from Shanghai Outdo Biotech Company (Cat. #HLugA180Su05). Samples collected from patients with concomitant central nervous system diseases or other types of malignant tumors were excluded from the microarray. Related information of all lung cancer patients was

collected and written informed consents were collected from each patient. This study was approved by The Ethical Committee of The First Affiliated Hospital of Nanchang University. For IHC, specimens were dewaxed in xylene and hydrated in ethanol. Then primary antibody *LMNB1* (Cat. #ab229025) was added for incubating at 4°C overnight, followed by a second antibody. Tissue specimens were stained with diaminobenzidine. The spots were pictured with a microscope and viewed by CaseViewer and ImageScope software. IHC score evaluation were performed based on the staining intensity and staining extent scores. For Ki-67 immunostaining, mice tumor tissues slides were incubated with antibody Ki-67 (Abcam, Cat. #Ab16667) and goat anti-rabbit IgG H&L (HRP) and stained with H&E (Baso, Cat. #BA4041, BA4022).

Mice Xenograft Model

Ten four-week-old, female BALB/c nude mice (Beijing Vitalriver Experimental Animal Technology Co., Ltd) were randomly divided into sh*LMNB1* and shCtrl group. 0.2 mL (2×10^7 cells/mL) stably transfected NCI-H1299 cell suspensions were injected into the right back of each mouse. Mice weight and tumor length and width were recorded every four days for six weeks. All mice were anesthetized by intraperitoneal injection of 0.7% sodium pentobarbital (10 µL/g) and the anesthetized mice were placed under a Perkin Elmer IVIS Spectrum (Waltham) for in vivo bioluminescence imagine. Finally, all mice were euthanized by injection of sodium pentobarbital (120 mg/kg body weight) and the tumors were removed. All animal experiments performed in our study were carried out at the Center of Laboratory Animal Science, Nanchang University, and approved by The Ethical Committee of The First Affiliated Hospital of Nanchang University.

Statistical Analysis

SPSS 18.0 software (Chicago, IL, USA) and GraphPad Prism 7.0 (La Jolla, CA, USA) were used for data analysis and $P < 0.05$ was considered statistically significant. Data in experiments were expressed as Means \pm SD and the significant differences were analyzed using Student's *t*-test. For analyzing the relations between patient's clinicopathological characteristics and *LMNB1* expressing, Fisher's exact, Spearman's rank correlation analysis and Mann-Whitney U analysis was applied. Kaplan-Meier method was used to plot survival analysis.

Results

Identification of *LMNB1* as Potential Tumor Promotor in LUAD

In order to explore key molecules in LUAD development, a global expression profiling was obtained through RNA-seq based on 10 tumor tissues and 10 normal tissues (Figure 1A). Among the DEGs upregulated in LUAD, 10 candidates, which have not been extensively studied were selected and abundant endogenous expression in NCI-H1299 cells were selected for further screening (Figure 1B). After delivering corresponding shRNAs into NCI-H1299 cells, the high-

content screening showed that knockdown of *LMNB1* showed the strongest effects on inhibiting LUAD cell proliferation (Figure 1C). Consistently, the detection of *LMNB1* in tumor and normal tissues by qPCR and IHC demonstrated the upregulated *LMNB1* expression in tumor tissues (Figure 1D-E, Table 1). Moreover, the statistical analysis revealed the significant correlation between *LMNB1* expression and pathological stage as well as lymphatic metastasis ($P < 0.05$, Tables 2 and 3). The Kaplan–Meier survival analysis significantly correlated high *LMNB1* expression with low survival rate (Figure 1F).

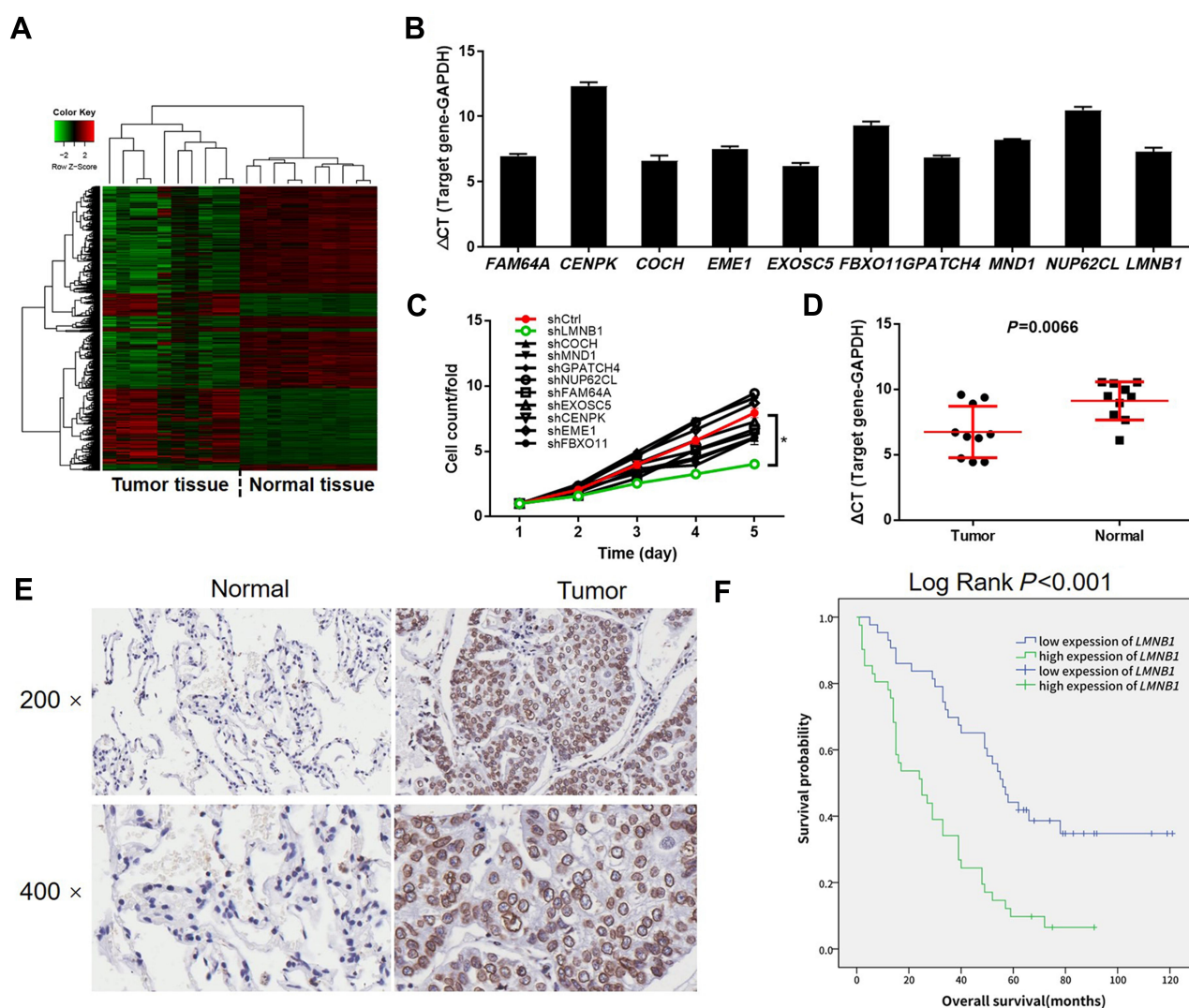


Figure 1 Identification of *LMNB1* as potential tumor promotor in LUAD. (A) RNA-seq was performed to identify differentially expressed genes (DEGs) in LUAD tumor tissues and normal tissues. (B) Endogenous expression of several selected DEGs was detected by qPCR in NCI-H1299 cells. (C) Celigo cell counting assay was utilized to evaluate the inhibition of cell proliferation by knockdown of the selected DEGs. (D, E) The expression of *LMNB1* in LUAD tumor tissues and normal tissues was detected by qPCR (D) and immunohistochemistry analysis (E). (F) Kaplan–Meier survival analysis was performed to show the correlation between *LMNB1* expression and prognosis of LUAD patients. * $P < 0.001$.

Table 1 Expression Patterns of *LMNB1* in LUAD Tissues and Normal Tissues Revealed in Immunohistochemistry Analysis

<i>LMNB1</i> Expression	Tumor Tissue		Normal Tissue	
	Cases	Percentage	Cases	Percentage
Low	44	51.2%	72	96%
High	42	48.8%	3	4%

Note: $P < 0.001$.

Depletion of *LMNB1* Inhibits Cell Proliferation and Migration, Induces Cell Apoptosis and Cell Cycle Arrest

Next, a loss-of-function study was performed to verify the role played by *LMNB1* in the development and progression of LUAD. Lentivirus transfection with efficiency of $>80\%$ was conducted to construct *LMNB1* knockdown cell lines, in which the knockdown of *LMNB1* was exerted by the most efficient shRNA (sh*LMNB1*-2, [Supplementary Figure S1](#)) and confirmed by qPCR and Western blotting, respectively ([Supplementary Figure S2](#) and [Figure 2A](#)). Celigo cell counting assay showed that cells with downregulated *LMNB1* possessed significantly lower proliferative activity ($P < 0.01$, [Supplementary Figure S3](#) and [Figure 2B](#)). Results of flow cytometry demonstrated that *LMNB1* knockdown induced cell apoptosis and cell cycle arrest in G2/M phase ($P < 0.01$, [Figure 2C-D](#)). Moreover, the reduced cell migration ability of LUAD cells with relatively lower expression of *LMNB1* was illustrated by both wound-healing and transwell assays ($P < 0.001$, [Figure 2E-F](#)). Collectively, all these outcomes indicated the role as a tumor promotor of *LMNB1* in LUAD.

Exploration of Mechanism by Which *LMNB1* Regulates LUAD

In order to explore the mechanism by which *LMNB1* knockdown inhibits LUAD development, an antibody microarray was performed to obtain expression profiling of apoptosis-related proteins ([Figure 3A](#)). As shown in [Figure 3B](#), statistical analysis demonstrated that pro-apoptotic proteins including Bax, Caspase3, Caspase8, Fas, FasL, IGFBP-4, p21 and p27 were upregulated, while anti-apoptosis proteins Bcl-2 was downregulated. Furthermore, based on the KEGG enrichment, expression of several key proteins in downstream enriched signaling pathways was detected by Western blotting, showing inhibited phosphorylation of Akt and suppressed expression of *CCND1*, *CDK6* and *PIK3CA* ([Figure 3C](#)).

Table 2 Relationship Between *LMNB1* Expression and Tumor Characteristics in Patients with LUAD

Features	No. of Patients	<i>LMNB1</i> Expression		P-value
		Low	High	
All patients	86	44	42	
Age (years)				0.391
≤61	43	20	23	
>61	43	24	19	
Gender				0.269
Male	48	22	26	
Female	38	22	16	
Tumor size				0.119
<4 cm	36	22	14	
≥4 cm	50	22	28	
Lymph node positive				0.006
≤1	47	30	17	
>1	36	12	24	
Grade				0.356
I	3	2	1	
II	56	30	26	
III	27	12	15	
Stage				0.012
I	25	18	7	
2	16	8	8	
3	39	14	25	
4	1	1	0	
T infiltrate				0.187
T1	16	11	5	
T2	49	23	26	
T3	15	9	6	
T4	6	1	5	
Lymphatic metastasis (N)				0.002
N0	36	24	12	
N1	14	7	7	
N2	16	5	11	
N3	4	0	4	
Expression of EGFR (FISH)				0.617
Negative	69	37	32	
Positive	11	5	6	

LMNB1 Knockdown Reduces Tumorigenesis of LUAD Cells in vivo

Finally, NCI-H1299 cells with or without *LMNB1* knockdown were inoculated into mice for constructing xenograft

Table 3 Relationship Between *LMNB1* Expression and Tumor Characteristics in Patients with LUAD Analyzed by Spearman Rank Correlation Analysis

Tumor Characteristics	Index	
Stage	Pearson's correlation	0.279
	Significance (two tailed)	0.012
	n	81
Lymphatic metastasis (N)	Pearson's correlation	0.365
	Significance (two tailed)	0.002
	n	70
Lymph node positive	Pearson's correlation	0.302
	Significance (two tailed)	0.005
	n	83

to present the role of *LMNB1* in the development and progression of LUAD. The measurement and calculation of tumor volume during animal culturing showed

significantly slower growth rate of xenografts formed by *LMNB1* knockdown cells ($P < 0.01$, Figure 4A). The in vivo imaging, weighing and direct observation of the xenografts all demonstrated smaller tumors formed in the sh*LMNB1* group (Figure 4B-D). The lower expression of Ki67 in tumor sections collected from the sh*LMNB1* group was in agreement with the lower proliferative activity (Figure 4E). Altogether, the outcome of the in vivo study revealed the inhibitory effects of LUAD induced by *LMNB1* knockdown.

Discussion

In recent years, lung cancer has been the most common malignant tumor in the world, and it is also the leading cause of cancer-related death.^{2,15} Among the various histological types of lung cancer, LUAD is the most frequently diagnosed subtype.¹⁶ At present, chemotherapy is still the major treatment for patients with advanced

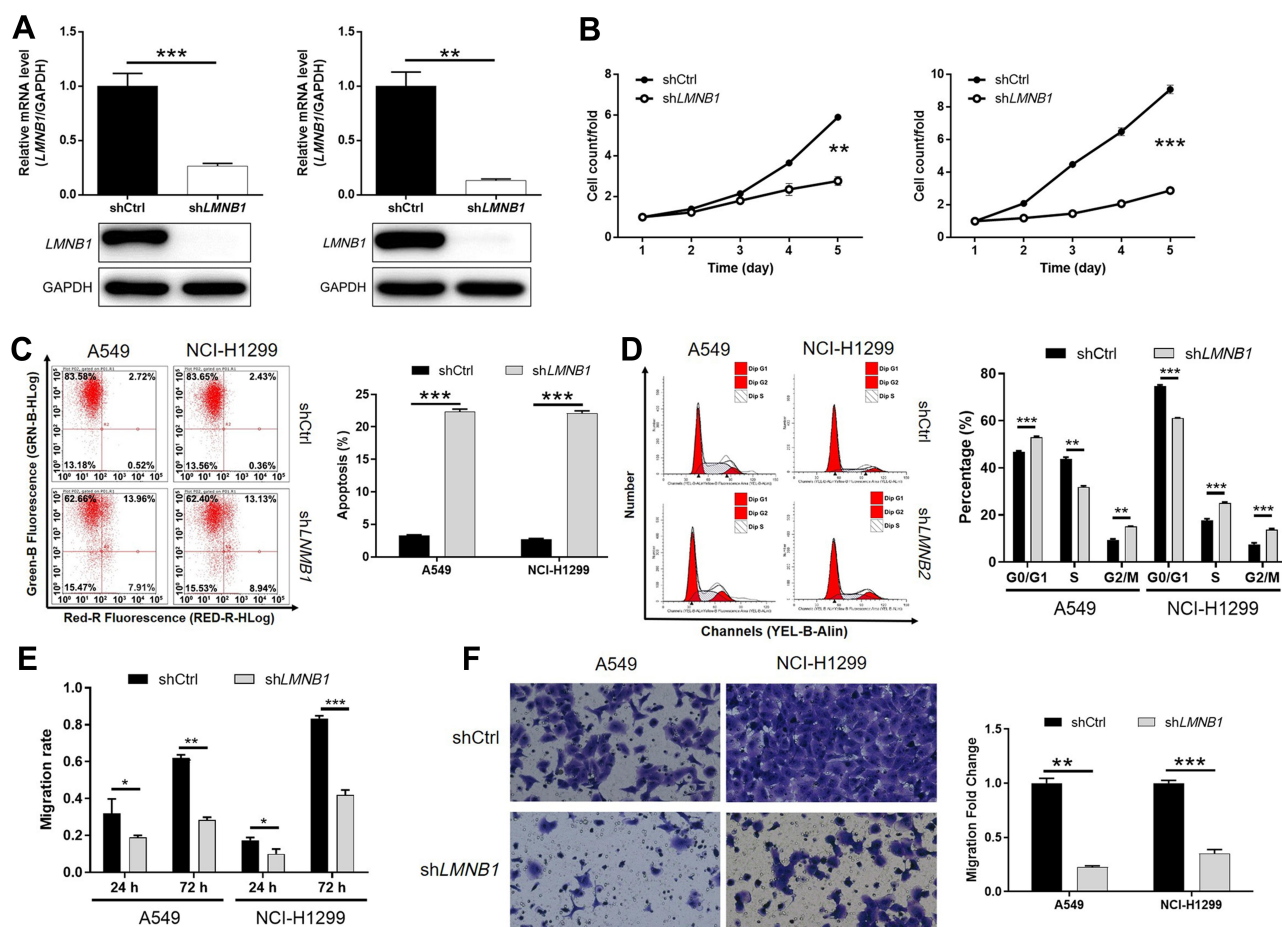


Figure 2 Depletion of *LMNB1* inhibits cell proliferation and migration, induces cell apoptosis and cell cycle arrest. (A) qPCR and Western blotting were performed to assess the knockdown efficiency of *LMNB1* in LUAD cells. (B) Effects of *LMNB1* knockdown on cell proliferation was determined by Celigo cell counting assay. (C, D) Influence of *LMNB1* depletion on cell apoptosis and cell cycle distribution was detected by flow cytometry. (E, F) Impacts of *LMNB1* knockdown on LUAD cell migration was examined by wound-healing (E) and transwell (F) assays. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

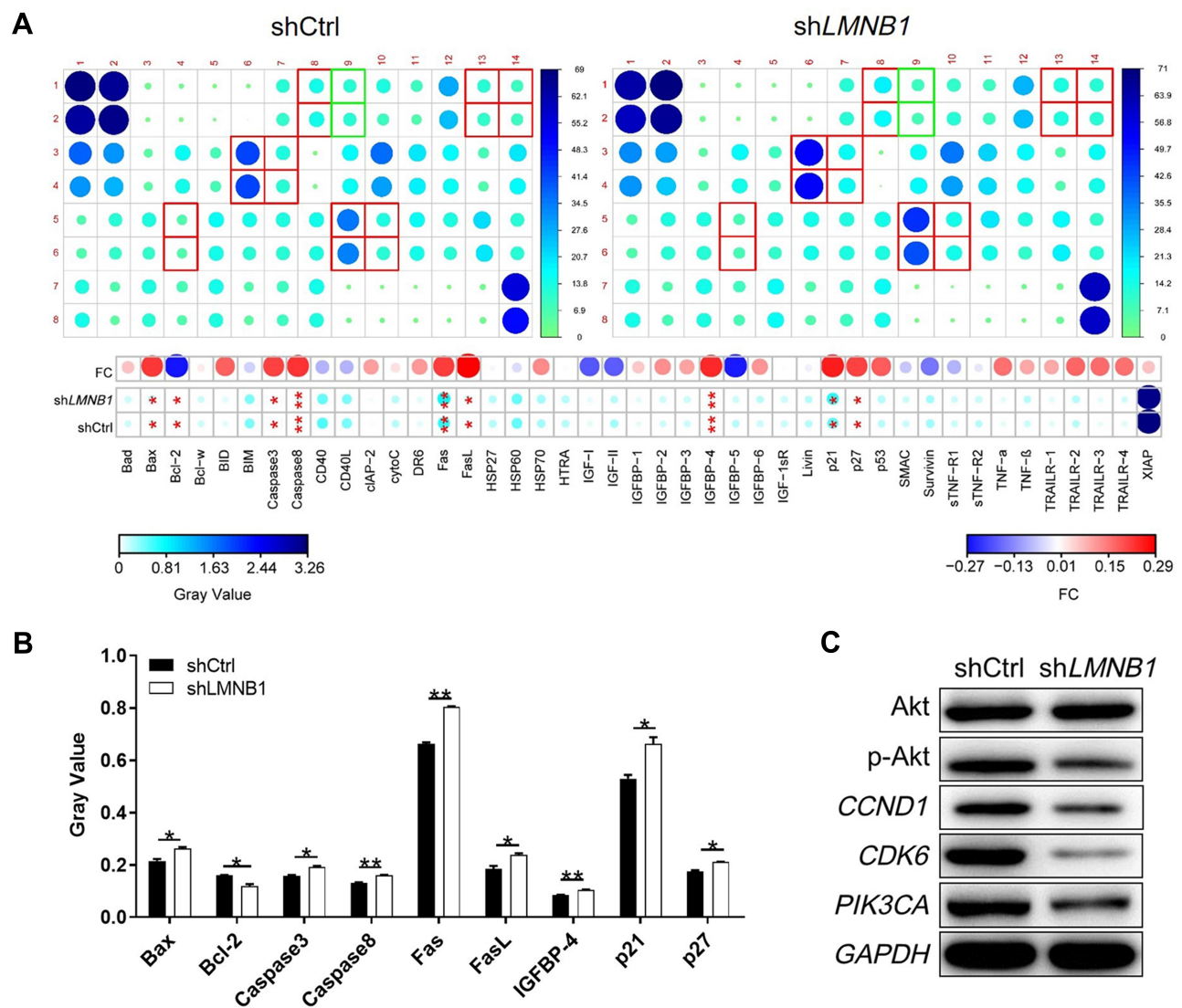


Figure 3 Exploration of mechanism by which *LMNB1* regulates LUAD. (**A**, **B**) Human apoptosis antibody array was performed to identify differentially expressed apoptosis-related proteins in NCI-H1299 cells with or without *LMNB1* knockdown. (**C**) Western blotting was performed to detect the changes in expression of proteins in cancer-related signaling pathways. * $P < 0.05$, ** $P < 0.01$.

LUAD, however, the therapeutic effects of which were seriously limited by the side effects and drug resistance.¹⁷ Although the development of various molecular targeted drugs targeting *EGFR*-TKI (such as gefitinib, afatinib and osimertinib),^{18,19} VEGF (such as bevacizumab and ramucirumab)²⁰ and *EGFR* (such as cetuximab)²¹ has improved the prognosis of LUAD to some extent,²² the exploration of novel therapeutic target of LUAD is still in urgent need and has attracted considerable attention.^{23,24}

LMNB1 is an important component protein of the nuclear skeleton. Although the biological function of *LMNB1* has been comprehensively studied, research concerning its role in human cancer is still rare. Izdebska et al

reported that *LMNB1* could induce dose-dependent cell death through mitotic catastrophe pathway, which may be a reason for fast development of colorectal cancer. They also revealed the correlation between *LMNB1* overexpression and poor clinical outcomes.²⁵ *LMNB1* was identified by Yang et al as target of traditional Chinese Medicine Huaier to mediate the inhibition of development and metastasis of prostate cancer.²⁶ Notably, *LMNB1* was recently found to be a tumor suppressor in lung cancer, loss of which promoted development and metastasis of lung cancer through recruiting polycomb repressive complex 2 (PRC2) thus activating RET/p38 signaling pathway.^{27,28} Interestingly, totally conversed results were obtained in our study.

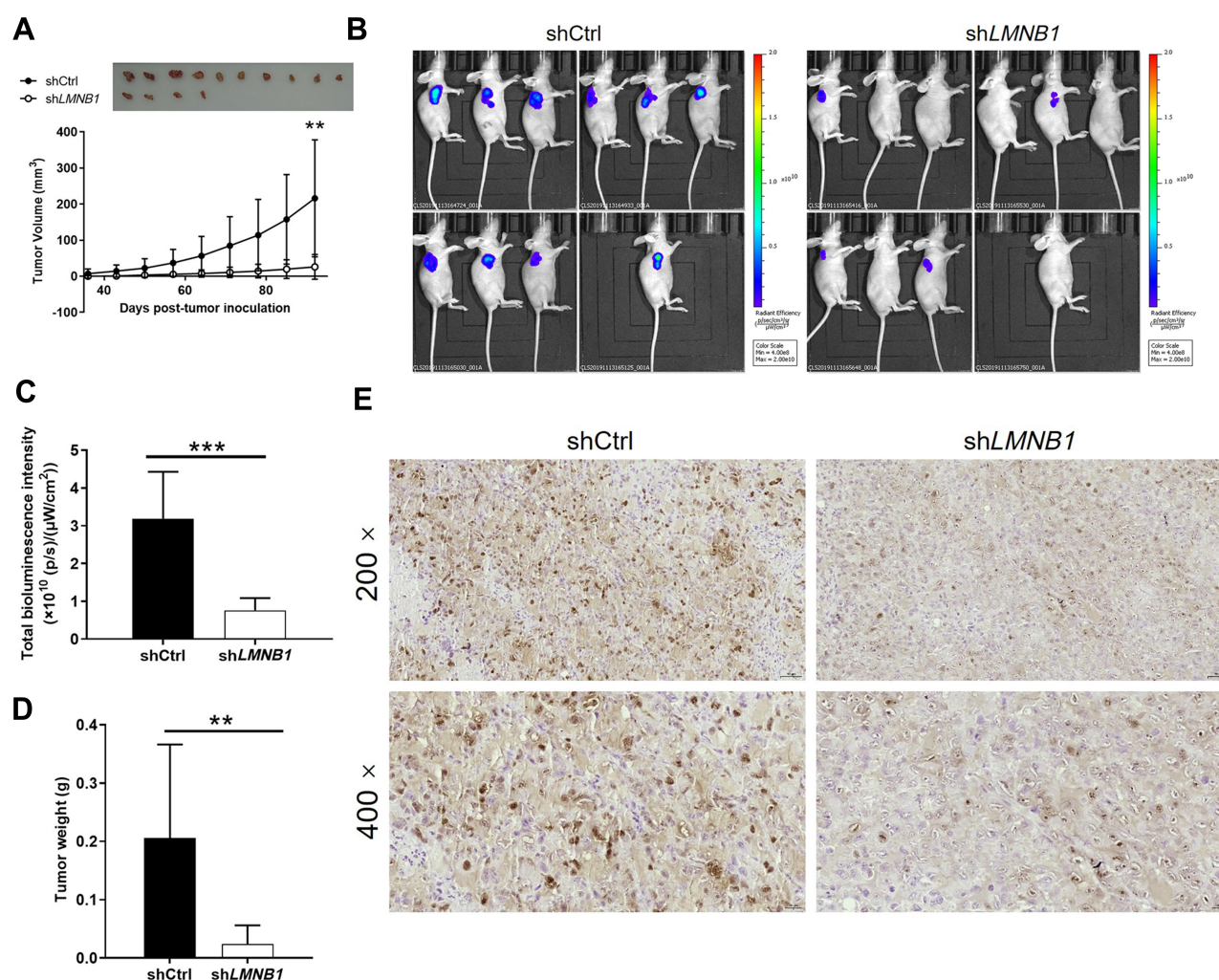


Figure 4 *LMNB1* knockdown reduces tumorigenesis of LUAD cells in vivo. **(A)** Tumor volume was calculated based on tumor size measured at indicated time intervals. Inset: photo of the removed xenografts. **(B)** In vivo imaging was performed to evaluate tumor growth. **(C)** The total intensity of bioluminescence was scanned to represent the tumor burden. **(D)** Tumor weight was measured after sacrificing mice. **(E)** The expression of Ki67 in tumor sections was detected by immunohistochemistry analysis. * $p < 0.01$, ** $p < 0.001$.

Herein, we found that *LMNB1* was upregulated in LUAD tumor tissues compared with normal tissues, high expression of which was significantly associated with more advanced tumor stage and higher risk of lymphatic metastasis. Following the verification of *LMNB1* high expression in lung cancer cell lines, A549 and NCI-H1299 cells with *LMNB1* knockdown were constructed through lentivirus-mediated shRNAs. The in vitro experiments indicated that *LMNB1* knockdown could significantly inhibit cell proliferation of A549 and NCI-H1299 cells, and induce cell apoptosis and cell cycle arrest in G2/M phase. In contrast, the suppressed cell migration ability of lung cancer cells in the shLMNB1 group revealed by both wound-healing and transwell assays suggested the potential involvement of *LMNB1* in metastasis of LUAD.

Furthermore, the results of in vitro studies were also verified in the mouse xenograft model, in which knockdown of *LMNB1* obviously slowed down the growth rate of tumors. All the results showed that *LMNB1* may act as a tumor promoter in the development of LUAD.

For revealing the mechanism underlying the regulatory effects of *LMNB1* on lung cancer, we also identified that the knockdown of *LMNB1* induced cell apoptosis of lung cancer cells through upregulating pro-apoptotic proteins including Bax, Caspase3, Caspase8, Fas, FasL, IGFBP-4, p21 and p27, and downregulating anti-apoptotic protein Bcl-2. It is well known that Akt-mediated signaling plays an important role in regulating the malignant behavior of tumor cells, such as proliferation, differentiation, apoptosis and migration.²⁹ In our study, it was found that the activity

of Akt, represented by its phosphorylation, was clearly suppressed by *LMNB1* knockdown. Moreover, well-known cancer-related proteins *CCND1*, *CDK6* and *PIK3CA* were also found to be downregulated by *LMNB1* knockdown, emphasizing the anticancer function of *LMNB1* knockdown again.^{30,31}

Conclusions

In conclusion, this study identified *LMNB1* as a tumor promotor in development and progression of lung cancer, knockdown of which could inhibit lung cancer through regulating cell proliferation, cell apoptosis, cell cycle and cell migration. *LMNB1* could probably be used as a novel therapeutic target in the treatment of lung cancer.

Ethics Approval

All animal experiments performed in our study were carried out at the Center of Laboratory Animal Science, Nanchang University in accordance with Guidelines for Animal Experiments of Animal Ethics Committee of Nanchang University, and approved by The Ethical Committee of The First Affiliated Hospital of Nanchang University.

Author Contributions

All authors made a significant contribution to the work reported, whether that was in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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