Lamin B2 contributes to the proliferation of bladder cancer cells via activating the expression of cell division cycle-associated protein 3

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Abstract. Bladder cancer is the most common malignant tumor of the urinary system, and in China it is first among urogenital system tumors. More therapeutic targets are still urgently required to combat this disease. Lamin B2 (LMNB2) is a type of nuclear lamina filament protein, which is involved in multiple cellular processes, and known as an oncogene affecting the progression of multiple types of cancers. Although the multiple effects of LMNB2 on cancer progression have been elucidated, its possible role in bladder cancer remains unclear. In the present study, it was determined that LMNB2 expression was upregulated in human bladder cancer tissues, and its expression was correlated with the prognosis and the clinical features, including tumor stage (P=0.001) and recurrence (P=0.006) of patients with bladder cancer. In addition, it was further revealed that LMNB2 depletion inhibited bladder cancer cell proliferation, stimulated cell cycle arrest and apoptosis *in vitro*, and suppressed tumor growth of bladder cancer cells in mice. Furthermore, the present data revealed that LMNB2 promoted the proliferation of bladder cancer cells via transcriptional activation of CDCA3 expression. Therefore, the role of LMNB2 in bladder cancer progression was demonstrated, and may serve as a promising therapeutic target for bladder cancer treatment.

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Introduction

Bladder cancer is the most common malignant tumor of the urinary system and is rated as the tenth most common form of cancer and the ninth leading cause of cancer-associated mortality worldwide in 2018, occurring in the mucous membrane of the bladder tissues (1,2). In China, its incidence ranks first among urogenital system tumors (3). Bladder cancer mainly includes bladder urothelial carcinoma, bladder squamous cell carcinoma and bladder adenocarcinoma (4). Early bladder cancer lacks significant symptoms, and a small number of patients develop hematuria, which is often in the advanced stage at the time of diagnosis (5). The main treatment methods for bladder cancer include surgical resection, chemoradiotherapy and targeted therapy, among which targeted therapy is the most effective and promising treatment for patients with advanced bladder cancer (6). To combat this disease and improve the prognosis of patients with bladder cancer, more therapeutic targets are still urgently needed.

Lamin B2 (LMNB2) is a type of nuclear lamina filament protein, which is involved in multiple cellular processes, such as transcription regulation and mitosis (7,8). During normal mitosis, LMNB2 regulates chromosome stability by ensuring proper mitotic chromosome segregation (9). Additionally, LMNB2 mediates nucleolar morphology, affects cardiomyocyte polyploidization, and promotes myocardial regeneration (10). It has also been reported that LMNB2 promotes retinal development, and the mutations of LMNB2 lead to neurodevelopmental and nuclear morphology defects (11,12). LMNB2 variants were revealed to cause primary microcephaly and define a novel laminopathy (12).

Notably, LMNB2 is recognized as an oncogene affecting the progression of multiple types of cancers, such as ovarian, lung, and liver cancer (13-15). LMNB2 was revealed to be aberrantly highly expressed in tumor tissues, and correlated with the prognosis of several cancers, such as breast, liver, and lung cancer (15-17). In a previous study, LMNB2 bound to MCM7 and promoted the activity of MCM7 helicase, thus promoting

the proliferation of lung cancer cells (16). Additionally, in another study, LMNB2 promoted the dimethylation of histone 3 lysine 9 (H3K9), and thus promoted the malignant phenotype of non-small cell lung cancer (NSCLC) (14). Although the multiple effects of LMNB2 on cancer progression have been elucidated, its possible role in bladder cancer remains unclear.

In the present study, the expression levels of LMNB2 in human bladder cancer tissues were assessed and its correlation with the prognosis and clinical features of patients was investigated. The involvement of LMNB2 in the regulation of bladder cancer cells *in vitro* and in mice was further demonstrated, and the molecular mechanism was clarified. It is therefore suggested that LMNB2 could serve as a promising therapeutic target for bladder cancer treatment.

Materials and methods

Bioinformatics analysis. Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn/) was used to analyze the data in The Cancer Genome Atlas (TCGA; https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) to determine the mRNA levels and the effects of LMNB2 and cell division cycle-associated protein 3 (CDCA3) on the survival rates of patients with bladder cancer. The expression of LMNB2 in several types of cancers was analyzed on the web of The Human Protein Atlas (https://www.proteinatlas.org/).

Human tissue samples. A total of 107 bladder cancer tissues and adjacent tissues were collected from patients who received surgical therapy at the College of Clinical Medicine of Henan University of Science and Technology (Luoyang, China). The present study was approved by the Ethics Committee of the College of Clinical Medicine of Henan University of Science and Technology (approval no. AMI-2020-073). The clinicopathological features of patients with bladder cancer, such as age, sex, tumor grade, and recurrence were analyzed and are presented in Tables I and II. The age distribution of the patients was 47-82 years (median 66 years).

The expression levels of LMNB2 and CDCA3 in tumor and adjacent tissues were detected using immunohistochemistry (IHC). All tissues were fixed in 4% formalin for 48 h at room temperature and embedded in paraffin. Subsequently, 4-μm sections were blocked using 5% BSA for 30 min at room temperature. The blocked sections were then incubated with primary antibodies: Anti-LMNB2 antibody (1:400; product code ab151735) and anti-CDCA3 antibody (1:200; product code ab166902; both from Abcam) for 2 h at room temperature, and subsequently incubated with a biotinylated secondary antibody kit (ready to use; cat. no. PV6000; ZSGB-BIO; OriGene Technologies, Inc.) for another 1 h at 37˚C according to the manufacturer's instructions. Finally, DAB solution was applied for color development.

The expression levels of LMNB2 and CDCA3 were manually divided based on the staining intensity (0, negative staining; 1, weak staining; 2, moderate staining; and 3 strong staining). Meanwhile, the proportion of stained cells was as follows (0, 0% positive-stained cells; 1, 1-30% positive-stained cells; 2, 31-60% positive-stained cells; and 3, 61-100% positive-stained cells). The total score was the staining intensity x the score of the percentage of positive-stained cells, and <1 or =1 was considered as negative staining, whereas 2-4 was considerate as weak staining and >4 was considered as strong staining of CDCA3.

The sections from each patient were observed in at least five light visual fields, and two experienced pathologists examined the sections using a light microscope at magnifications of x100 (scale bar, 100 μm) and x200 (scale bar, 50 μm).

Cell culture and transfection. The human bladder cancer cells, including T24 (ATCC no. HTB-4) and 5637 cells (ATCC no. HTB-9), and normal epithelial cell SV-HUC-1 (ATCC no. CRL-9520) were all obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM; cat. no. 11971025; Thermo Fisher Scientific, Inc.) supplemented with 10% of fetal bovine serum (FBS; cat. no. 04-001-1A; Biological Industries), 1% penicillin-streptomycin (cat. no. P1400; Beijing Solarbio Science & Technology Co., Ltd.) and incubated at 37˚C in a 5% CO_2 incubator.

The indicated plasmids in the present study were transfected into bladder cancer cells using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). LMNB2 knockdown was confirmed in both T24 and 5637 cells, two of the most commonly used *in vitro* models for bladder cancer.

All sequences were synthesized by Sangon Biotech Co., Ltd. (https://www.sangon.com/). The shRNA plasmids of LMNB2 and CDCA3 were constructed in our laboratory. Short hairpin (sh)RNA LMNB2, 5'-GCAGAGTTGGACGAG GTCAACAAGA-3'; shRNA CDCA3, 5'-CCGCTCTCCTAC TCTTGGTATTGCA-3'; and shRNA scramble, 5'-ACTCAA GAGTCTAGCAAGCCTGCAG-3'. The overexpression plasmids of pcDNA3.1-vector (cat. no. 34706) were obtained from Addgene, Inc. and pcDNA3.1-LMNB2 and pcDNA3.1-CDCA3 plasmids were constructed in our laboratory. Other plasmids, pGL3-Basic Luciferase Reporter Vector (cat. no. E1751; Promega Corporation) and pGL-CDCA3 plasmids were constructed in our laboratory. For plasmid transfection, 2.5 μg plasmid ($OD_{260/280}$=1.8-2.0) were transfected into target cells ($50x10^4$) using Lipofectamine 3000 (cat. no. L3000001; Thermo Fisher Scientific, Inc.) for 15 min at room temperature. After 2 days, subsequent experimentations were performed.

For lentivirus transduction, the plasmid PLKO.1 (3rd generation; cat. no. 10878; Addgene, Inc.) was used to produce the lentivirus according to the manufacturer's instructions. Simply, 6 μg pMDL (cat. no. 12251), 3 μg pVSV-G (cat. no. 138479), 2 μg pRSV-Rev (cat. no. 12253; all from Addgene, Inc.) and 5 μg PLKO.1 were transfected into 293FT cells (80% confluence; cat. no. R70007; Thermo Fisher Scientific, Inc.) using Lipofectamine® 3000 (cat. no. L3000001; Thermo Fisher Scientific, Inc.) for 15 min at room temperature. After 2 days, the lentivirus was harvested from the supernatant by ultracentrifugation at 72,000 x g at 4˚C for 120 min. Subsequently, $2x10^5$ T24 and 5637 cells were transfected with the lentivirus for 36 h at 37˚C and 5% CO_2, with an MOI of 1:5. After 3 days, LMNB2-knockdown cells were selected using 2 μg/ml puromycin (cat. no. P8230; Beijing Solarbio Science & Technology Co., Ltd.) for 5 days.

Reverse transcription-quantitative (RT-q)PCR assays. Total RNA was extracted from bladder cancer cells using TRIzol reagent (cat. no. 15596-018; Invitrogen; Thermo Fisher Scientific,

Table I. Associations between the expression of LMNB2 and clinicopathological characteristics in 107 patients with bladder cancer.

Features	Total no. of patients (n=107)	LMNB2 expression		χ^2	P-value
		Low n=32	High n=75		
Age (years)				0.824	0.364
<65	54	14	40		
≥65	53	18	35		
Sex				0.077	0.782
Male	58	18	40		
Female	49	14	35		
Tumor stage				10.449	0.001[a]
T2	36	18	18		
T3/T4	71	14	57		
Tumor grade				1.222	0.269
Low	29	11	18		
High	78	21	57		
Lymph node metastasis				1.195	0.274
Yes	20	8	12		
No	87	24	63		
Recurrence				7.422	0.006[a]
Yes	55	10	45		
No	52	22	30		

[a]P<0.05. LMNB2, Lamin B2.

Inc.). Then total RNA was reverse-transcribed using M-MLV reverse transcriptase kit (cat. no. M1701; Promega Corporation) according to the manufacturer's instructions. RT-qPCR was then performed using SYBR Green mixture (cat. no. RR420A; Takara Bio, Inc.). The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 3 min; followed by 30 cycles of denaturation at 95˚C for 30 sec, annealing at 58˚C for 30 sec and extension at 72˚C for 30 sec. The $2^{-\Delta\Delta Cq}$ method was used to quantify the results (18). LMNB2 and CDCA3 expression levels were normalized to the expression of GAPDH. The following primers were used:

LMNB2 forward, 5'-TTTCCACCAACAGGGGGAC-3' and reverse, 5'-ACGTTCTGGCAGTTCGCTT-3'; CDCA3 forward, 5'-CACCTAGTGCTGGCATCCTG-3' and reverse, 5'-GGCAGAACAGGCTCTCCACT-3'; GAPDH forward, 5'-ATGGGCAGCCGTTAGGAAAG-3' and reverse, 5'-GCCCAATACGACCAAATCAGAGA-3'.

Western blot analysis. Bladder cancer cells were lysed using RIPA buffer (product no. 9806S; Cell Signaling Technology, Inc.). All the cell and tissue samples were isolated to extract the proteins and determined using the BCA method. A total of 30 μg protein per lane was separated by 10% SDS-PAGE, sequentially transferred onto the PVDF membranes, followed by blocking with 5% fat-free milk in TBST buffer at room temperature for 30 min. PVDF membranes were then treated with primary antibodies targeting a series of proteins at room temperature for 1.5 h. Subsequently the membranes were incubated with secondary antibodies which were HRP-conjugated at room temperature for 1 h. The secondary antibodies were as follows: Goat anti-rabbit secondary antibody (cat. no. ZB-2301; 1:10,000; ZSGB-BIO; OriGene Technologies, Inc.) and goat anti-mouse secondary antibody (cat. no. G-21040; dilution, 1:10,000; Thermo Fisher Scientific, Inc.). Signals were detected using an ECL kit (Novex ECL Chemiluminescent Substrate Reagent kit; Thermo Fisher Scientific, Inc.) and analyzed using ImageJ software (v1.32; National Institutes of Health) according to the manufacturer's protocol. The primary antibodies used for the various experiments were as follows: Anti-LMNB2 antibody (1:400 dilution for IHC; 1:1,500 dilution for western blotting; and 1:200 dilution for CHIP assays; product code ab151735), anti-CDCA3 antibody (1:200 dilution for IHC; 1:2,000 dilution for western blotting; product code ab166902), anti-Ki67 antibody (1:1,000 dilution; product code ab16667), anti-proliferating cell nuclear antigen (PCNA) antibody (1:500 dilution; product code ab29), anti-caspase-3 antibody (1:1,000 dilution; product code ab32351), anti-caspase-8 antibody (1:1,000 dilution; product code ab32397), and anti-β-actin antibody (1:3,000 dilution; product code ab8226; all from Abcam).

Colony formation assay. A total of 1,000 bladder cancer cells were re-seeded into 6-well plates in complete medium and

Table II. Associations between the expression of CDCA3 and clinicopathological characteristics in 107 patients with bladder cancer.

Features	Total no. of patients (n=107)	CDCA3 expression		χ^2	P-value
		Low n=35	High n=72		
Age (years)				2.280	0.131
<65	54	14	40		
≥65	53	21	32		
Sex				0.704	0.402
Male	58	21	37		
Female	49	14	35		
Tumor stage				7.368	0.007[a]
T2	36	18	18		
T3/T4	71	17	54		
Tumor grade				1.328	0.249
Low	29	7	22		
High	78	28	50		
Lymph node metastasis				0.594	0.441
Yes	20	8	12		
No	87	27	60		
Recurrence				10.853	0.001[a]
Yes	55	10	45		
No	52	25	27		

[a]P<0.05. CDCA3, cell division cycle-associated protein 3.

maintained for nearly 2 weeks in a cell incubator, until the colonies were formed. Subsequently, the colonies were fixed with 4% paraformaldehyde (PFA) for 20 min and stained with 0.1% crystal violet at room temperature for 20 min. Following staining, colonies (>1 mm^2 was considered a cell colony) were captured and analyzed by ImageJ (ImageJ 1.48v; National Institutes of Health).

MTT assay. Bladder cancer cells were seeded into 96-well plates with 5,000 cells/well and maintained for 12 h. On days 1, 2, 3, 4 and 5, cells were subsequently incubated with MTT for 2 h and then washed with PBS. Cells were then isolated using 150 μl DMSO and the OD_{570} was analyzed.

Cell Counting Kit-8 (CKK-8) assay. Bladder cancer cells were plated into 96-well plates with a density of 5,000 cells and subsequently maintained for 12 h. On days 1, 2, 3, 4 and 5, cells were then treated with 10 μl CCK-8 (cat. no. 96992; Merck KGaA) for 2 h and the OD value was measured at 490 nm.

Cell cycle assay. Bladder cancer cells ($1x10^6$) were fixed using 70% ethylalcohol for 24 h at -20˚C and then incubated with a concentration of 100 μg/ml propidium iodide (PI) at 37˚C for 15 min. Subsequently the samples (300 μl) were analyzed using FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (FlowJo v10.6.2; FlowJo LLC). The percentage of cells at different phases was compared.

Cell apoptosis assay. Bladder cancer cells ($1x10^6$) were re-suspended and incubated with Annexin V-FITC and propidium iodide kit (cat. no. V13242; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 10 min according to the manufacturer's instructions. Subsequently, the samples were analyzed using FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (FlowJo v10.6.2; FlowJo LLC), and the apoptosis of cells in different groups was analyzed and compared.

Tumor growth in vivo assay. The procedures used for the animal assays were all approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Clinical Medicine of Henan University of Science and Technology (approval no. SYXK 2020-0127). The female BALB/c nude mice (8 weeks old; weight, 19-21 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All mice in the present study were fed *ad libitum* with food and water, and were maintained in specific pathogen-free conditions at 20˚C, a 12-h light/dark cycle and 60% humidity. A total of 10 nude mice were used in the present study, including control (n=5) and LMNB2-knockdown mice (n=5). To measure tumor growth *in vivo*, T24 cells were stably transfected with LMNB2 shRNA plasmids and injected into the right flank of female nude mice. After 7 days, tumors began to be established, and the volume of the tumors was assessed every 7 days and calculated. After 42 days, the tumor growth

curves were calculated and compared between different groups. The tumor volume was calculated as follows: Tumor volume (mm^3)=tumor length (mm) x tumor width $(mm)^2/2$. Humane endpoints were used during the animal assays and the mice were sacrificed if they met one of humane endpoints: Weight loss of >25%, maximum tumor diameter >20 mm. Mice were sacrificed by intraperitoneal injection of sodium pentobarbital (100 mg/kg). Mortality was confirmed by cervical dislocation. The maximum tumor volume observed in the xenograft study was 700 mm^3.

ChIP and luciferase assays. ChIP assays were performed using a CHIP Assay kit (product code ab500; Abcam). A total number of $1x10^8$ T24 cells were crosslinked, resuspended and lysed, then sonicated so as to shear the DNA into a range of 600-900 bp. The chromatin fraction was then immunoprecipitated using LMNB2 (1:200 dilution; product code ab151735) or IgG targeted antibodies (1:200 dilution; product code ab172730; both from Abcam), respectively, and the mix was enriched by the use of protein A Agarose (product code ab193254; Abcam). Beads were isolated and washed five times. DNA was finally purified and quantified using Nanodrop spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies; Thermo Fisher Scientific, Inc.). Next-generation sequencing (300 bp paired-end sequencing) and data analysis were then conducted at Sangon Biotech Co. Ltd. Briefly, 20 pm DNA was used for sequencing using NovaSeq 6000 SP reagent kit (100 cycles; cat. no. 2002746; Illumina Inc.). Quality-control and data analysis were performed using FastQC software (version 0.11.9; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), Bowtie2 (version 2.2.4; http://bowtie-bio.sourceforge.net/bowtie2/index.shtml), and R (version 4.0.5; https://www.r-project.org/).

For luciferase assays, bladder cancer cells were maintained and transfected with 2.5 μg pGL-CDCA3 (cat. no. E1751; Promega Corporation), pGL-Basic, pEnter-LMNB2 plasmids for 48 h using Lipofectamine 3000. The cells were then washed twice and lysed, and the luciferase activities were then detected using Dual-Luciferase Reporter Assay System (cat. no. E1910; Promega Corporation) according to the manufacturer's protocol. *Renilla reniformis* luciferase activity was measured to normalize transfection efficiency.

Statistical analysis. GraphPad 6.0 (GraphPad Software, Inc.) was used for all statistical analyses. Data were expressed as the mean ± SEM in the present study. The survival analysis between clinical features and LMNB2 expression was performed using Kaplan-Meier method with log-rank testing test. Student's t-test was used for statistical comparisons between control and shRNA groups, and multiple comparisons were performed using ANOVA and post hoc Tukey's test. Spearman's and Pearson's correlation analyses were performed to analyze the associations of LMNB2 and CDCA3. The experiments were repeated at least three times.

Results

LMNB2 is upregulated in human bladder cancer tissues and correlated with the prognosis and clinical features of patients. According to the bioinformatics data from GEPIA database, it was observed that LMNB2 was widely expressed in multiple types of tumor tissues, such as thyroid cancer, glioma, and colorectal cancer (Fig. 1A), and associated with the prognosis of patients with several types of cancers, including renal, prostate, liver, and stomach cancer (Fig. 1B). These data suggested LMNB2 was a potential tumor regulator.

To clarify whether LMNB2 could affect the progression of bladder cancer, the expression levels of LMNB2 in human bladder cancer tissues and normal tissues were first investigated using GEPIA database. A total of 404 tumor tissues and 28 normal tissues were included in this comparison. It was observed that the mRNA levels of LMNB2 in tumor tissues were obviously higher than those in normal tissues (Fig. 1C). Subsequently, the effects of LMNB2 on the prognosis of patients was further investigated using GEPIA database, and it was revealed that LMNB2 was significantly associated with the disease-free survival rates of patients in two cohorts (19,20) (P=0.023 and P=0.045, respectively; Fig. 1D), suggesting an association between LMNB2 and prognosis. Therefore, it was determined that LMNB2 was upregulated in human bladder cancer tissues and associated with the prognosis of patients.

The expression levels of LMNB2 in 107 human bladder cancer tissues and the corresponding adjacent tissues collected at our hospital were then detected using IHC assays. Similar to the results obtained in the GEPIA database, it was observed that LMNB2 expression was significantly higher in tumor tissues than that in adjacent tissues (Fig. 2), further confirming that LMNB2 was upregulated in human bladder cancer tissues.

Subsequently, the patients were divided into two groups, including LMNB2 low and high expression groups, according to the expression levels of LMNB2 in tumor tissues. It was observed that 32 (32/107; 29.9%) patients exhibited LMNB2 low expression, whereas the remaining (75/107, 70.1%) exhibited high expression of LMNB2 in tumor tissues (Table I). To further clarify the associations between LMNB2 expression and the clinical features of patients, the age, sex, tumor stage, tumor grade, lymph node metastasis, and recurrence of disease in patients were investigated. No evident associations between LMNB2 expression and clinical features including age (P=0.364), sex (P=0.782), tumor grade (P=0.269), and lymph node metastasis (P=0.274; Table I) were found. However, it was determined that the expression of LMNB2 was significantly associated with the tumor stage (P=0.001) and recurrence of disease (P=0.006; Table I) of patients with bladder cancer. Collectively, our data confirmed that LMNB2 was upregulated in human bladder cancer tissues and associated with the prognosis and clinical features of patients.

LMNB2 depletion impairs the proliferation of bladder cancer cells via stimulating cell cycle arrest and apoptosis in vitro. Subsequently, it was determined that LMNB2 was overexpressed in bladder cancer cells, T24 and 5637 cells, compared with normal epithelial cell line, SV-HUC-1 (Fig. 3A). It was then investigated whether LMNB2 could affect the proliferation of bladder cancer cells *in vitro*. The shRNA plasmids of LMNB2 were constructed and stably transfected into two types of human bladder cancer cells, including T24 and 5637 cells, to deplete its expression. Through RT-qPCR assays, it was demonstrated that the transfection of LMNB2 shRNA plasmids could significantly decrease the mRNA levels of

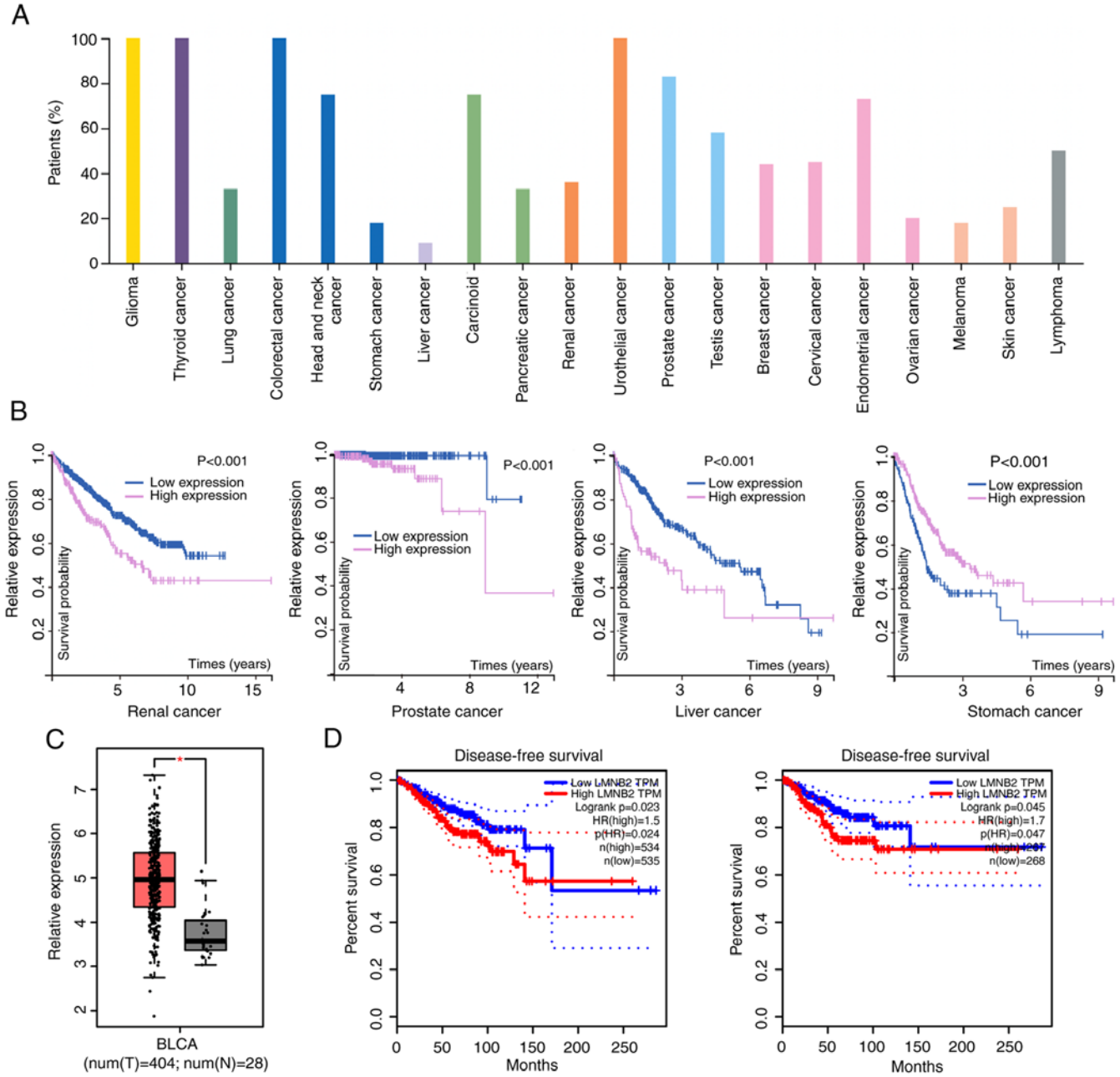

Figure 1. LMNB2 mRNA levels are upregulated in human bladder cancer tissues and associated with the prognosis of patients. (A) The Human Protein Atlas database revealed LMNB2 was highly expressed in several types of cancers, such as thyroid cancer and glioma, and (B) associated with the survival rates of patients with several cancers, such as liver cancer and stomach cancer. (C) GEPIA database revealed that LMNB2 mRNA levels were upregulated in 404 bladder cancer tissues compared with in those in 28 normal tissues. (D) LMNB2 expression levels were associated with the disease-free survival rates (P=0.024 and P=0.047, respectively) of two sets of bladder cancer patients, confirmed by GEPIA database. Results are presented as the mean ± SEM. *P<0.01. LMNB2, Lamin B2; GEPIA, Gene Expression Profiling Interactive Analysis.

LMNB2 in T24 and 5637 cells (Fig. 3B). Similarly, it was established that the protein expression of LMNB2 was significantly decreased upon the transfection of LMNB2 shRNA plasmids in both T24 and 5637 cells, as confirmed by western blot analysis (Fig. 3C).

Notably, using MTT and CCK-8 assays, it was determined that the knockdown of LMNB2 in T24 and 5637 cells decreased the OD value at 570 and 490 nm, respectively, suggesting the impairment of bladder cancer cell proliferation (Fig. 3D and E). It was then detected whether LMNB2 affected the proliferation of bladder cancer cells via colony formation assays. The results revealed that LMNB2 ablation significantly suppressed the proliferation of T24 and 5637 cells, as observed by the decreased number of colonies (Fig. 3F). Furthermore, consistent with the previous results found, through western blotting, it was observed that the expression levels of two proliferation markers, Ki67 and PCNA, were significantly decreased after LMNB2 depletion in T24 and 5637 cells, suggesting the inhibition of cell proliferation (Fig. 3G and H).

Next, it was determined whether LMNB2 affected bladder cancer cell proliferation via mediation of the cell cycle or cell apoptosis through flow cytometric assays. According to the results, it was revealed that LMNB2 depletion led to cell cycle arrest with an increase in the number of cells at the G2

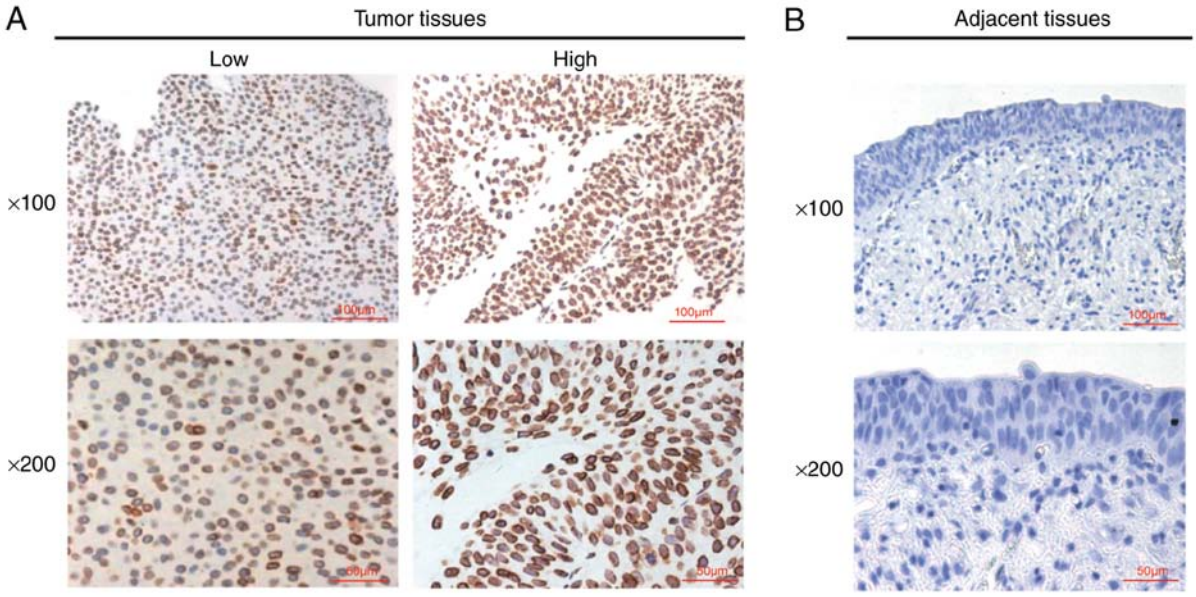

Figure 2. LMNB2 protein levels were upregulated in bladder cancer tissues obtained from patients with bladder cancer. (A) Representative images of LMNB2 expression detected through IHC assays in human bladder cancer tissues (x100 and x200 magnification, respectively). (B) LMNB2 expression levels are shown in the corresponding normal tissues (x100 and x200 magnification, respectively) through IHC assays. LMNB2, Lamin B2; IHC, immunohistochemistry.

phase in T24 and 5637 cells (Fig. 4A). Additionally, through flow cytometric assays, it was determined that the knockdown of LMNB2 promoted the apoptosis of bladder cancer cells, with an increase in the number of early or late apoptotic cells (Fig. 4B). Furthermore, knockdown of LMNB2 in bladder cancer cells increased the expression of caspase-3 and caspase-8 (Fig. 4C). Subsequently, overexpression of LMNB2 in SV-HUC-1 cells demonstrated that LMNB2 promoted cell proliferation and impaired cell apoptosis as observed by the decrease in the expression of caspase-3 and caspase-8 (Fig. 4D-F). Therefore, these data provided the evidence that LMNB2 depletion suppressed the proliferation of bladder cancer cells via stimulating cell cycle arrest and apoptosis.

LMNB2 promotes the tumor growth of bladder cancer cells in mice. To further confirm the effects of LMNB2 on bladder cancer progression, *in vivo* assays were performed. Nude mice were subcutaneously injected with T24 cells stably-transfected with control or LMNB2 shRNA plasmids. After 7 days, the tumor volume was measured every 7 days. The representative images from each group were captured and are presented in Fig. 5A. After 42 days, all the tumors were isolated, and the growth curves were calculated and compared between two groups. Consistent with the *in vitro* data, the tumors from LMNB2-depleted cells were significantly smaller than those in the control groups, according to the growth curves (Fig. 5A). LMNB2 expression in tumor tissues was then detected using western blotting. The results revealed that the expression levels of LMNB2 in the LMNB2-depleted tissues were significantly decreased compared with the control (Fig. 5B). Therefore, these results indicated that LMNB2 depletion suppressed tumor growth of bladder cancer cells in mice.

LMNB2 contributes to bladder cancer progression via promotion of CDCA3 expression. The molecular mechanisms underlying LMNB2 promotion of bladder cancer progression were then investigated. Through bioinformatics analysis, it was observed that LMNB2 expression was positively correlated with the expression of CDCA3 in bladder cancer tissues (Fig. 6A). As has been reported, CDCA3 is widely involved in the regulation of multiple types of cancers (21,22). In addition, through the GEPIA database and IHC staining, it was determined that CDCA3 mRNA and protein expression were upregulated in bladder cancer tissues compared with normal and adjacent tissues, respectively (Fig. 6B and C).

The associations between CDCA3 expression and the clinical features of these 107 bladder cancer patients were then investigated. Similarly, it was revealed that CDCA3 expression was also significantly associated with tumor stage (P=0.007) and recurrence of disease (P=0.001) of bladder cancer patients (Table II). It was therefore theorized that LMNB2 promoted bladder cancer progression via the promotion of CDCA3 expression.

Through RT-qPCR assays, it was determined that the mRNA level of CDCA3 was increased in the LMNB2-overexpressed T24 cells, and decreased in the LMNB2-depleted T24 cells (Fig. 7A). Notably, the mRNA expression of LMNB2 was not affected in the CDCA3-overexpressed or depleted T24 cells (Fig. 7B).

To further clarify whether LMNB2 promoted bladder cancer cell proliferation through the promotion of CDCA3 expression, rescue assays were performed. The colony formation assays revealed that the cell proliferation caused by LMNB2 overexpression was markedly reversed by CDCA3 depletion (Fig. 7C). In addition, the cell proliferation was confirmed by MTT and CCK-8 assays, revealing that LMNB2 regulated cell proliferation through CDCA3 (Fig. 7D and E). These data confirmed that LMNB2 promoted bladder cancer cell proliferation via CDCA3.

Through ChIP assays, it was revealed that the antibody of LMNB2 could be specifically co-immunoprecipitated by the promoter fragment of CDCA3 in T24 cells, whereas

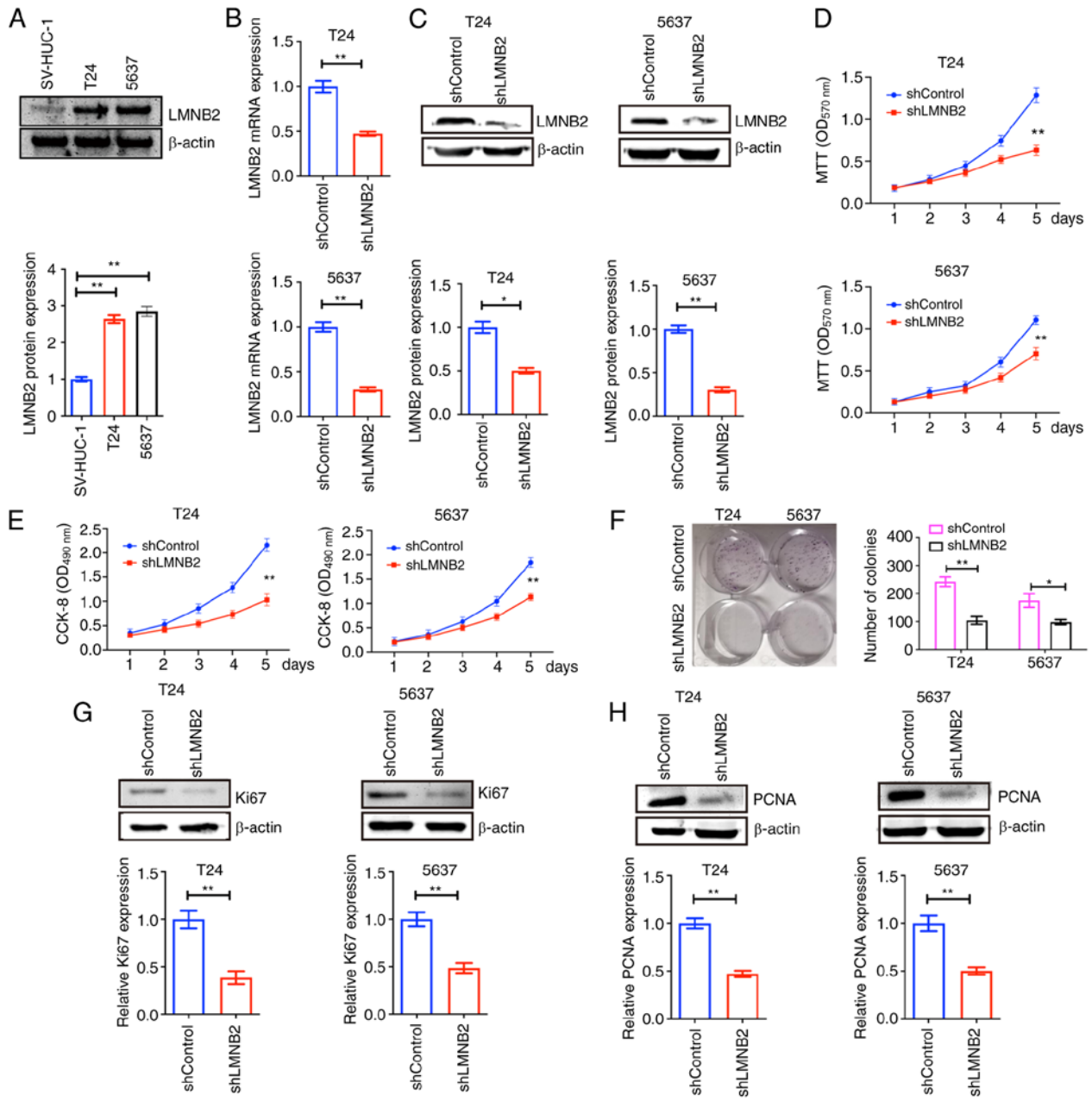

Figure 3. LMNB2 depletion impairs the proliferation of bladder cancer cells. (A) Western blotting revealed that LMNB2 was overexpressed in T24 and 5637 cells compared with normal epithelial cells, SV-HUC-1. (B) RT-qPCR revealed that LMNB2 expression was efficiently decreased after the transfection of its shRNA plasmids in T24 and 5637 cells. (C) Western blotting revealed that LMNB2 expression was decreased in T24 and 5637 cells upon the stable transfection of LMNB2 shRNA plasmids. (D) MTT assays revealed the OD value at a 570-nm wavelength of T24 and 567 cells transfected with control or LMNB2 shRNA plasmids. (E) CCK-8 assays revealed the OD value at a 490-nm wavelength of bladder cancer cells upon the transfection of the indicated shRNA plasmids. (F) Colony formation assays revealed the proliferation capacity of T24 and 5637 cells transfected with control or LMNB2 shRNA plasmids. (G and H) Western blotting showed the expression levels of Ki67 and PCNA in control or LMNB2 shRNA-transfected T24 and 5637 cells. Results are presented as the mean ± SEM. *P<0.05 and **P<0.01. LMNB2, Lamin B2; RT-qPCR, reverse transcription-quantitative PCR; shRNA, short hairpin RNA; OD, optical density; CCK-8, Cell Counting Kit-8; PCNA, proliferating cell nuclear antigen.

the IgG antibody could not be co-immunoprecipitated, suggesting the specific binding of LMNB2 with CDCA3 promoter (Fig. 7F). Furthermore, by performing luciferase assays, the pGL-CDCA3 plasmids containing the promoter region of CDCA3 were co-transfected with pEnter-vector or pEnter-LMNB2 plasmids into T24 cells. It was determined that LMNB2 could promote the activation of CDCA3 promoter in T24 cells (Fig. 7G). Therefore, it was further revealed that LMNB2 promoted bladder cancer progression via transcriptionally activating CDCA3.

LMNB2 cooperates with CDCA3 to promote bladder cancer progression. The aforementioned data revealed that LMNB2 could bind to CDCA3 promoter and transactivate CDCA3, and therefore promoted bladder cancer progression. IHC assays were then performed, and the expression levels of both LMNB2 and CDCA3 in 107 human bladder cancer tissues were detected and analyzed in continuous slices of tumor tissues. Notably, it was revealed that the expression of CDCA3 was markedly associated with the expression levels of LMNB2 (P=0.003; Fig. 8 and Table III). It was therefore concluded that

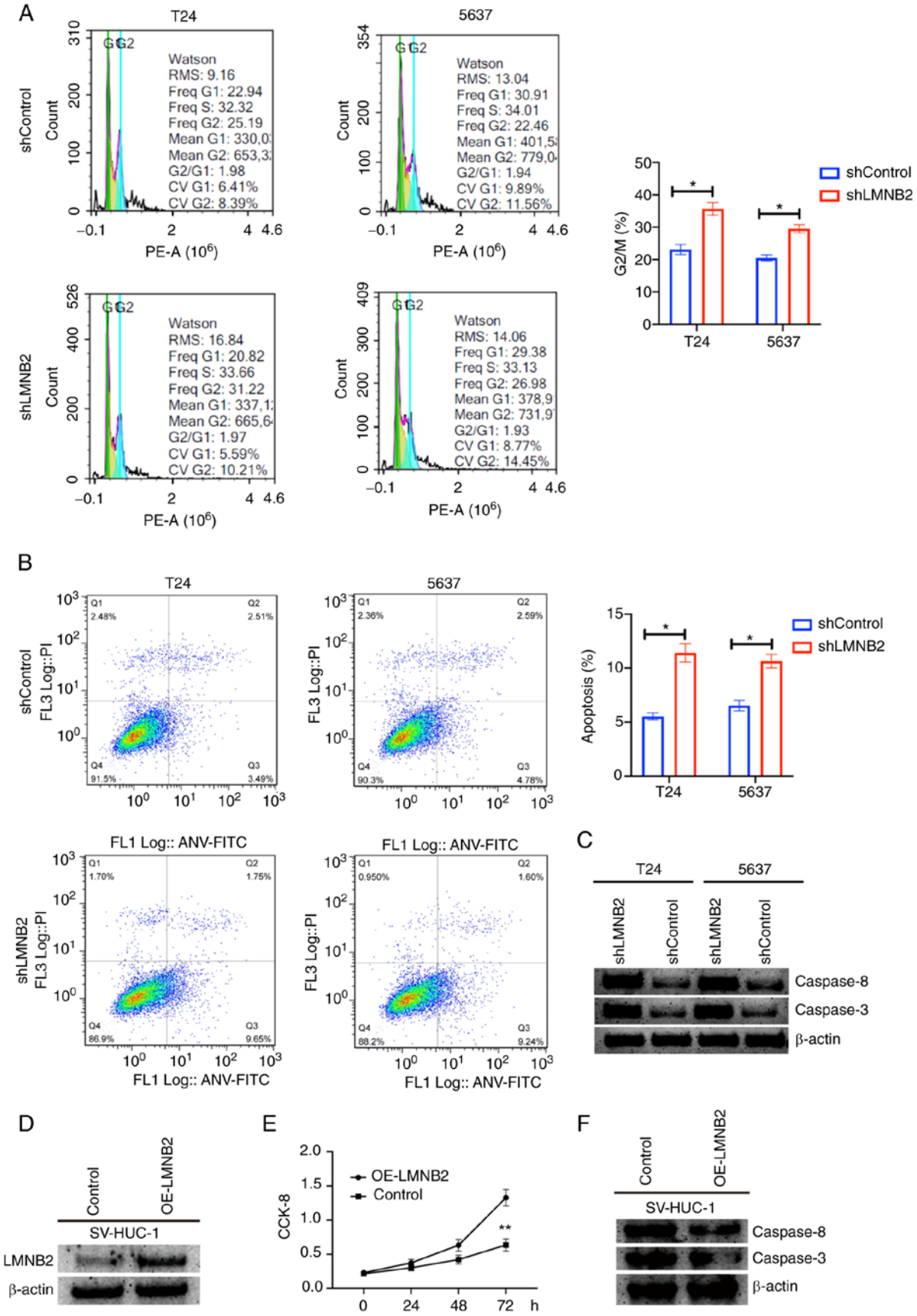

Figure 4. LMNB2 depletion impairs the proliferation of bladder cancer cells via stimulating cell cycle arrest and apoptosis *in vitro*. (A) T24 and 5637 cells were transfected with control or LMNB2 shRNA plasmids, and flow cytometric assays were performed. The percentage of cells at the G1, S, and G2/M phases was detected. (B) T24 and 5637 cells were stained with Annexin-FITC/PI, and flow cytometric assays were performed. The percentage of apoptosis of cells was detected. (C) The protein expression of caspase-3 and caspase-8. (D) LMNB2 was overexpressed in SV-HUC-1 cells, and the protein levels of LMNB2 were detected using western blotting. (E) CCK-8 assay and (F) caspase protein detection in control SV-HUC-1 cells and OE-LMNB2 SV-HUC-1 cells. Results are presented as the mean ± SEM. *P<0.05 and **P<0.01. LMNB2, Lamin B2; shRNA, short hairpin RNA; CCK-8, Cell Counting Kit-8; OE, overexpressed.

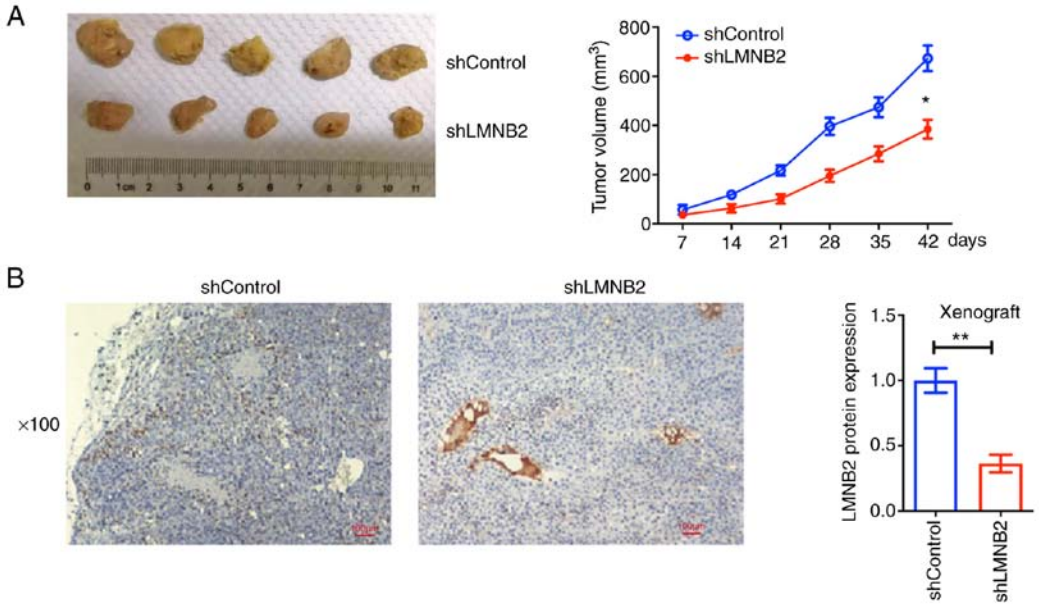

Figure 5. LMNB2 contributes to the tumor growth of bladder cancer cells in mice. (A) Representative images of 5 tumors isolated from nude mice formed by T24 cells stably-transfected with control or LMNB2 shRNA plasmids (n=5 in each group). After 7 days, tumor formation began, and the volume of tumors was measured every 7 days. (B) IHC assays revealed the expression levels of LMNB2 in control or LMNB2-depleted tumor tissues. Results are presented as the mean ± SEM. *P<0.05 and **P<0.01. LMNB2, Lamin B2; shRNA, short hairpin RNA; IHC, immunohistochemistry.

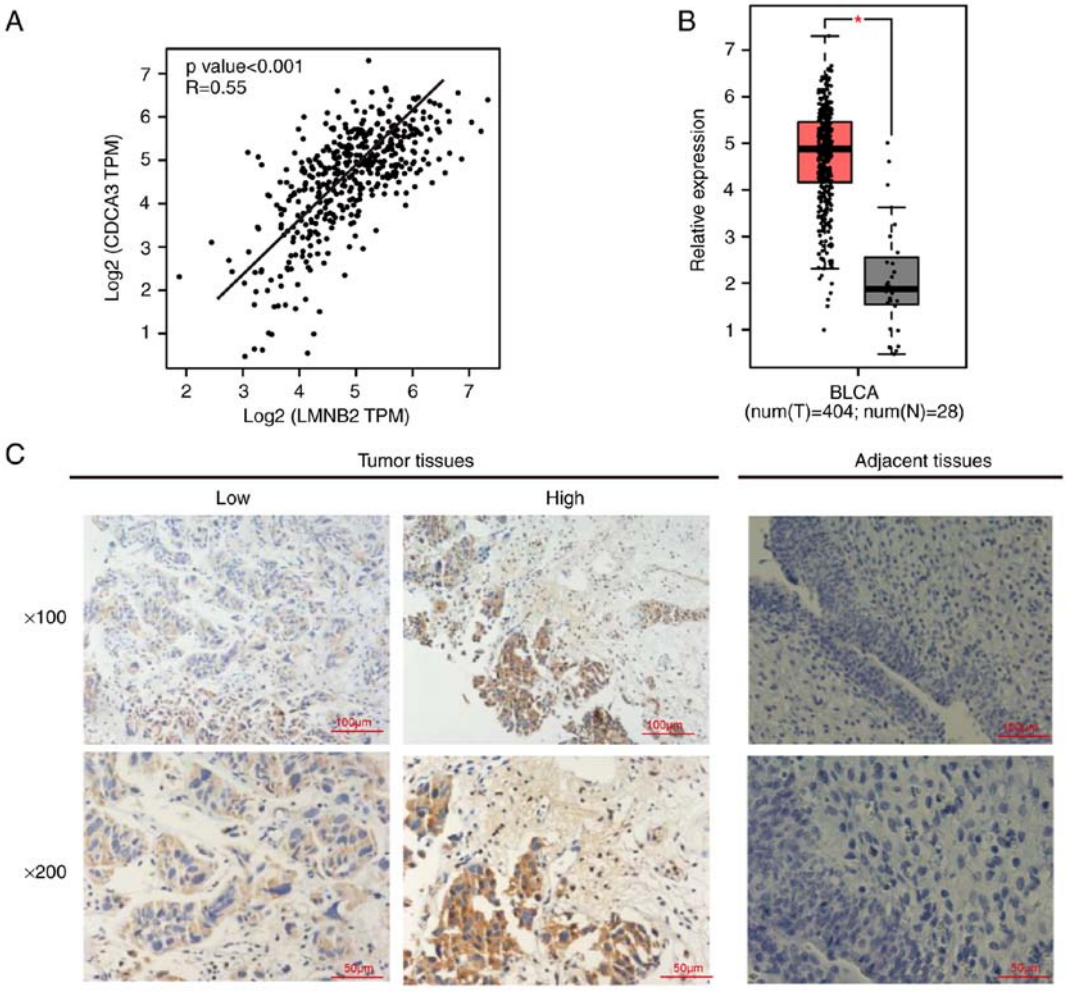

Figure 6. LMNB2 is positively correlated with the expression of CDCA3 in bladder cancer. (A) GEPIA database revealed the correlation analysis between the expression of LMNB2 and CDCA3 in bladder cancer tissues. (B) GEPIA database showed that CDCA3 mRNA levels were upregulated in 404 bladder cancer tissues compared with 28 normal tissues. *P<0.05. (C) The IHC staining of CDCA3 in bladder cancer tissues and normal tissues. LMNB2, Lamin B2; CDCA3, cell division cycle-associated protein 3; IHC, immunohistochemistry.

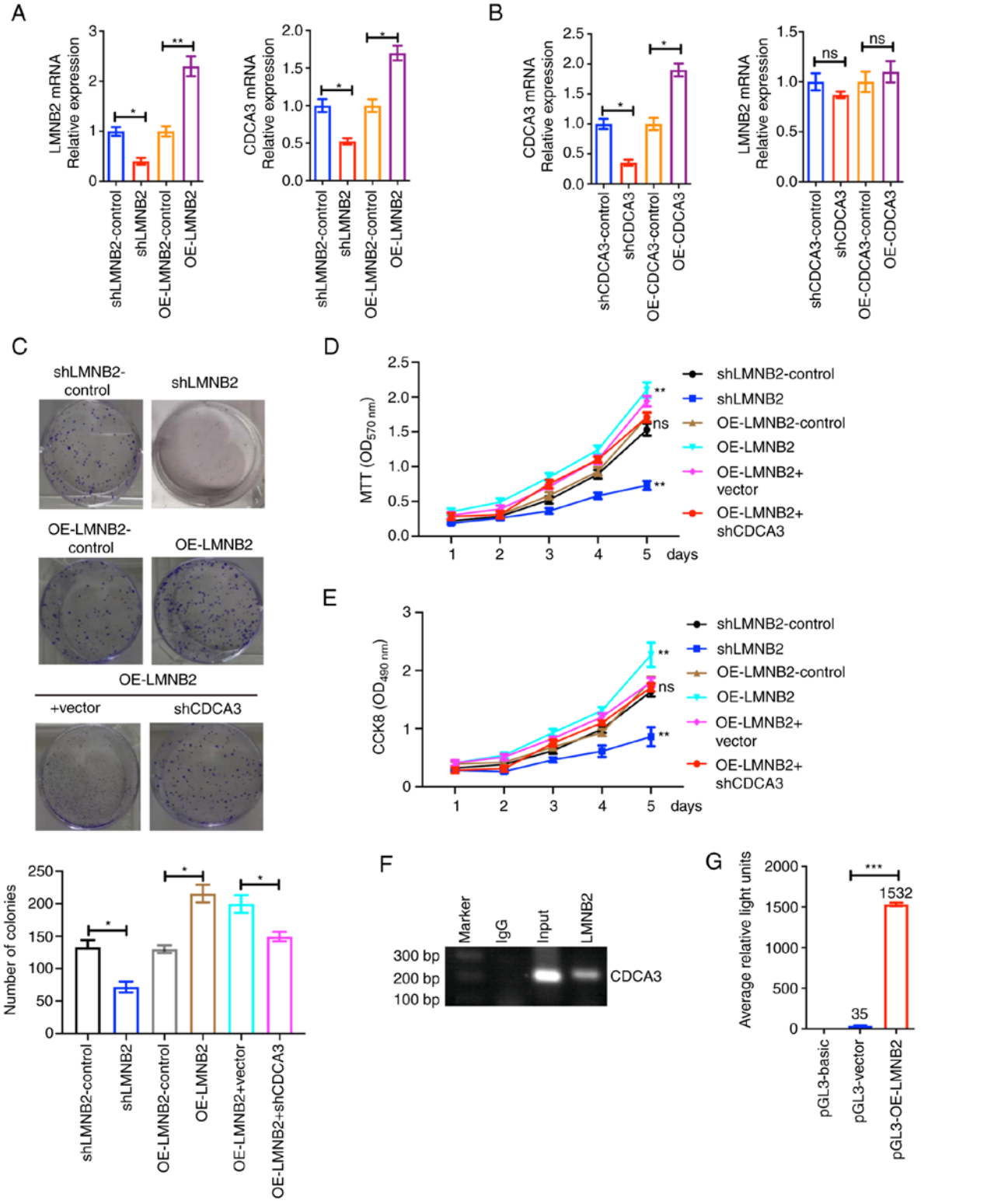

Figure 7. LMNB2 promotes the proliferation of bladder cancer cells via transcriptional activation of CDCA3. (A and B) LMNB2 and CDCA3 expression levels in T24 cells upon the transfection of the indicated plasmids were detected by RT-qPCR assays. (C) Colony formation assays showed the proliferation capacity of T24 cells upon the transfection of the indicated plasmids. (D and E) MTT and CCK-8 assays showed the OD values at 570 and 490 nm wavelengths, respectively, of T24 cells with the transfection of the indicated plasmids. (F) ChIP assays were performed, and PCR amplification was used after the enrichment of CDCA3 promoter fragment by the anti-IgG or anti-LMNB2 antibody in T24 cells. (G) The luciferase activity of pGL3-Basic and pGL3-CDCA3 in T24 cells co-transfected with pcDNA3.1-LMNB2 or pcDNA3.1-vector plasmids was measured by luciferase reporter assays. Results are presented as the mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001. LMNB2, Lamin B2; CDCA3, cell division cycle-associated protein 3; RT-qPCR, reverse transcription-quantitative PCR; CCK-8, Cell Counting Kit-8; sh, short hairpin; OE, overexpressed.

LMNB2 promoted the progression of bladder cancer through transcriptional activation of CDCA3. Collectively, it was determined that LMNB2 promoted the progression of bladder cancer through the promotion of CDCA3 expression.

Table III. Associations between LMNB2 and CDCA3 expression in 107 patients with bladder cancer.

Total no. of patients (n=107)	LMNB2		χ^2	P-value	Pearson	Spearman
	Low (n=32)	High (n=75)				
CDCA3			8.644	0.003	0.284	0.284
Low (n=35)	17	18				
High (n=72)	15	57				

LMNB2, Lamin B2; CDCA3, cell division cycle-associated protein 3.

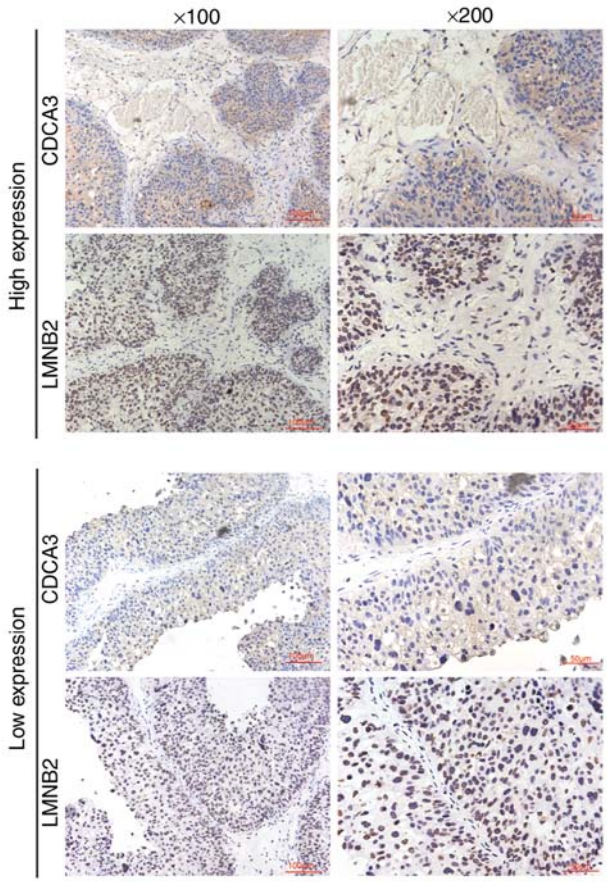

Figure 8. Expression of CDCA3 is associated with LMNB2 in human bladder cancer tissues. IHC assays were performed and revealed CDCA3 and LMNB2 expression in human bladder cancer tissues (x100 and x200 magnification, respectively). CDCA3, cell division cycle-associated protein 3; LMNB2, Lamin B2; IHC, immunohistochemistry.

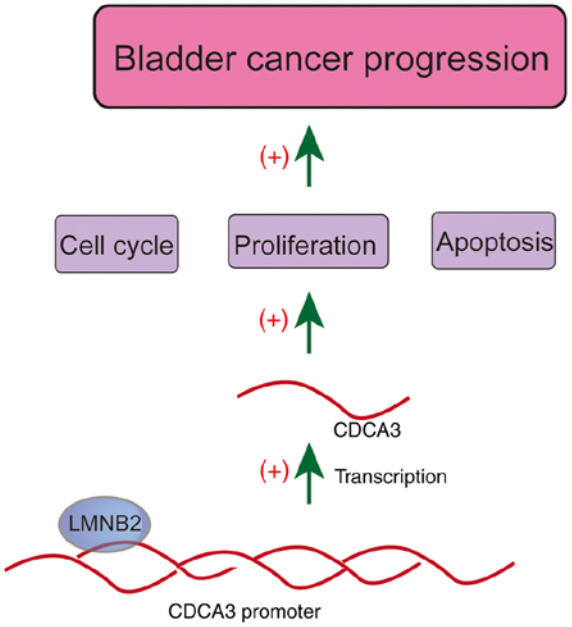

Figure 9. Regulatory mechanism of LMNB2 in the progression of bladder cancer. LMNB2 transcriptionally activates CDCA3 expression, and therefore mediates the cell cycle, apoptosis, and cell proliferation, and further promotes the progression of bladder cancer. LMNB2, Lamin B2; CDCA3, cell division cycle-associated protein 3.

Discussion

Early diagnosis and targeted therapy of bladder cancer are very important to improve the prognosis of patients with bladder cancer (23-25). The key of targeted therapy is to elucidate the pathogenesis of bladder cancer and explore new therapeutic targets (26). In the present study, the high expression of LMNB2 in human bladder cancer tissues was revealed. The expression of LMNB2 was associated with the prognosis and clinical features of bladder cancer patients. Our data further confirmed that LMNB2 affected the cell cycle and apoptosis, and promoted the proliferation of bladder cancer cells *in vitro* and *in vivo* via transcriptional activation of CDCA3 (Fig. 9). The present data therefore provided the evidence that LMNB2 could serve as a promising molecular target for bladder cancer treatment.

As is known, LMNB2 is ubiquitously expressed in all types of cells and developmental stages (27). The defects and mutations of LMNB2 have led to several types of diseases (28). A previous study indicated that the mutations of LMNB2 led to acquired partial lipodystrophy (28). Another study provided evidence that the mutation of LMNB2 stimulated progressive myoclonus epilepsy with early ataxia (29). These studies suggested the critical function of LMNB2 in development. In the present study, the involvement of LMNB2 in the progression of bladder cancer was demonstrated, however the precise regulatory mechanism requires further study. LMNB2 has also been shown to localize to mitochondria in axons, and therefore suppressed axonal degeneration via maintaining mitochondrial function and axonal integrity (30). In the future, whether LMNB2 regulated bladder cancer progression via regulating mitochondrial function will be studied.

It has also been widely reported that LMNB2 affects the progression and metastasis of multiple types of cancers (15,16). LMNB2 was also aberrantly highly expressed in several cancer tissues, including liver and lung cancer (15,16). LMNB2 protein levels were correlated with the prognosis and survival rates of patients with lung cancer, similar to the results of our study (31). Additionally, LMNB2 also affected the proliferation of lung cancer cells via binding MCM7 and promoting MCM7 helicase activity (16). In the present study it was determined that LMNB2 promoted the proliferation of bladder cancer cells. It was also revealed that LMNB2 affected the cell cycle and apoptosis of bladder cancer cells. The present data indicated that LMNB2 affected the proliferation of bladder cancer cells via regulation of CDCA3 expression. Therefore, it is suggested that LMNB2 could act as a therapeutic target for bladder cancer treatment.

CDCA3 is also known as a critical cancer regulator in multiple types of cancers (21,22). It has been reported that CDCA3 affected tumor formation in pancreatic cancer, and promoted cell proliferation in gastric and lung cancer (21,32,33). In colorectal cancer, CDCA3 could mediate p21-dependent proliferation via promoting E2F1 expression (22). In addition, in colorectal cancer, CDCA3 promoted cell proliferation via activating the NF-κB pathway (34). Notably, CDCA3 was correlated with the TNM staging and prognosis of patients with bladder cancer (35). Our hypothesis that LMNB2 promoted bladder cancer progression via regulation of CDCA3 expression is considered to be feasible. Limitedly, the binding site of LMNB2 regulating CDCA3 mRNA expression was not been revealed in the present study.

In summary, the aberrant expression of LMNB2 in human bladder cancer tissues, and that its expression was associated with the prognosis and clinical features of patients with bladder cancer was revealed. LMNB2 depletion resulted in the impairment of cell proliferation, the arrest of the cell cycle, and the promotion of apoptosis in bladder cancer cells. It was further confirmed that LMNB2 promoted the proliferation of bladder cancer *in vitro* and *in vivo* via transcriptional activation of CDCA3. It is therefore considered that LMNB2 could serve as a promising therapeutic target for the treatment of bladder cancer.

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Availability of data and materials

The datasets supporting the conclusions of this study are included within the manuscript.

Authors' contributions

JJ and WW conceived and designed the study, and confirmed the authenticity of all the raw data. HL collected the patient data. JJ and JC acquired the data. JC analyzed the data. JJ wrote and reviewed the manuscript. WW supervised the study. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All applicable hospital guidelines for the care and use of animals were followed. The use of animals was approved (approval no. SYXK 2020-0127) by the Ethics Committee of the College of Clinical Medicine of Henan University of Science and Technology. All procedures performed in studies involving human participants were in accordance with the ethical standards of the hospital research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The present study was approved (approval no. AMI-2020-073) by the Ethics Committee of the College of Clinical Medicine of Henan University of Science and Technology. Preoperative patients signed informed consent for the use of postoperative specimens for scientific research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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