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Knockdown of Lumican Inhibits Proliferation and Migration of Bladder Cancer

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Abstract

Lumican (LUM) is differentially expressed between normal and cancer tissues. The purpose of this study was to investigate the role of LUM in the proliferation and migration of bladder cancer (BCa) cells. Our study included 97 cases of BCa diagnosis from our hospital between June 2013 and June 2016. The expression of LUM was analyzed by immunohistochemistry and Western blot. To characterize the function of LUM, BCa cells were stably infected with a lentivirus against LUM, and cell proliferation, migration and cell cycle were investigated. In addition, xenograft experiments were performed in nude mice to evaluate the role of LUM in BCa. Our results showed that LUM was overexpressed in BCa tissues and cell lines in comparison to normal tissues. LUM expression was related to pathological type, T stage and N stage ($P < .05$). In addition, depletion of LUM inhibited the proliferation and migration of BCa cells by inactivating MAPK signaling. In conclusion, LUM promotes the proliferation and migration of BCa cells and may serve as a potential therapeutic target for BCa.

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Introduction

Bladder cancer (BCa) is the second most common malignancy in the urinary system after prostate cancer in the United States. In 2018, BCa had an estimated 81,190 new cases diagnosed and 17,420 cancer deaths [1]. The major histological type of BCa is urothelial carcinoma, of which approximately 75% are non-muscle invasive bladder cancers (NMIBC) and approximately 25–30% are muscle invasive bladder cancers (MIBC) [2,3]. Transurethral resection of bladder tumors (TURBT) is the main treatment for NMIBC. However, the 5-year recurrence rate of NMIBC is approximately 50–70% [4,5]. Radical cystectomy with pelvic lymph node dissection (RC/PLND) remains the standard treatment for patients with MIBC, with a total postoperative survival rate of approximately 60% [6]. Therefore, it is still imperative to find new potential therapeutic targets.

Lumican (LUM) belongs to the family of small leucine-rich proteoglycans (SLRPs), and the gene is located at the distal end of the human chromosome 12, consisting of 3 exons and 2 introns with a total length of approximately 6.9 kb [7,8]. In recent years, it has been found that LUM is not only expressed in intervertebral disc, skin, lung, liver skeletal muscle and other tissues but also presents an abnormal expression in various malignant tumors, such as breast, colon and pancreatic cancer [9–12]. One study has shown that LUM was highly expressed in BCa tissues [8], but its role in tumor occurrence, proliferation and migration was not further studied.

In this study, we investigated the expression of LUM in patients with BCa and further analyzed the effect of LUM on the proliferation and migration of BCa cells.

Patients and Methods

Tissue Samples

Between June 2013 and June 2016, we collected 97 samples from eligible surgically treated BCa tissue and adjacent normal tissue. None of

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the patients in this study received any radiotherapy or chemotherapy before the surgery. Fresh tissues were fixed in 4% paraformaldehyde for IHC staining or stored in liquid nitrogen. The tumors were classified according to the 2009 version of the American Joint Committee on Cancer/Union for International Cancer Control tumor, lymph node and metastasis (TNM) staging system. This study was approved by the Ethics Committee of Shanghai Tenth People's Hospital of Tongji University and written informed consent was obtained from all patients or their relatives.

Cell Lines

Human BCa T24, J82 and TCC-SUP cell lines and the normal bladder epithelial cell line Sv-HUC were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Sv-HUC cells were cultured in F12K medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), while the T24, J82 and TCC-SUP cell lines were maintained in RPMI 1640 or DMEM medium (Gibco; Thermo Fisher Scientific, Inc.). All media were supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (HyClone; GE Healthcare Life Sciences, Logan, UT, USA). All cell lines were cultured in an incubator containing 5% CO_2 at 37 °C.

Immunohistochemistry (IHC)

Fresh human bladder tissue samples and tumor tissue samples from nude mice were fixed in 4% paraformaldehyde, dehydrated in an ethanol solution and embedded in paraffin. IHC was performed as previously described [13]. The sections were incubated with an antibody against LUM (Abcam, Cambridge, MA) and imaged at the appropriate magnification under the microscope (Leica Microsystems, Mannheim, Germany).

Western Blot

Total protein was extracted from tissues or cells. Protein lysates (50 μg/lane) were separated by 10% sodium lauryl sulfate-polyacrylamide gels (SDS-PAGE) and transferred onto a nitrocellulose membrane (Sigma-Aldrich; Merck KGaA). The membranes were blocked in 5% nonfat milk for 1 hour at room temperature and incubated at 4 °C overnight with a primary antibody: anti-LUM, anti-ERK, anti-p-ERK, anti-MEK1/2, anti-p-MEK1/2 and anti-MMP-2 (Abcam, Cambridge, MA), anti-Cyclin D1 (BOSTER, Wuhan, China). β-actin (Abcam, Cambridge, MA) was used as an internal control. After incubation with the secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 hour at room temperature, the signals were visualized using the LI-COR Image Studio lite imaging system.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from the cultured cells by Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions, and the corresponding cDNA was generated using a cDNA synthesis kit (Takara Biotechnology, Dalian, China). Quantitative real-time PCR (qRT-PCR) was performed using the KAPA SYBR FAST qPCR kit and the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Relative expression of LUM was determined and compared to internal standards using the $2^{-\Delta\Delta Ct}$ method. The primers used in this study were as follows: LUM forward, 5′-GGATTGGTAAACCTGACCTTCAT-3′ and reverse, 5′-GATAAACGCAGATACTGCAATGC-3′; GAPDH forward, 5′-CAGGAGGCATTGCTGATGAT-3′ and reverse, 5′-GAAGGCTGGGGCTCATTT-3′.

Transfection

Specific small interfering RNA probes targeting human LUM (si-LUM) and si-NC were synthesized from RiboBio Corporation (Guangzhou, China). si-LUM and si-NC oligonucleotides were transiently transfected into T24 and J82 cell lines using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). Lentiviral molecules harboring si-LUM and si-NC sequences were constructed by DiduBio Corporation (Suzhou, China) and transfected according to the manufacturer's protocol.

Cell Proliferation

For the cell proliferation assay, transfected cells were seeded into 96-well plates at a density of 2000 cells per well. At 0, 24, 48, 72 and 96 hours after plating, 10 μl Cell Counting Kit-8 (CCK-8; Yeasen, Shanghai, China) solution was added to each well, and the 96-well plate was incubated in darkness for 2 hours at 37 °C. The optical density of each well was measured at 450 nm using a microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

Cell Cycle Analysis

T24 and J82 cells were harvested and washed twice with cold PBS 48 hours after transfection, and then the cells were fixed in 70% ethanol overnight at 4 °C. The fixed cells were washed with PBS and stained with RNase A (Sigma-Aldrich) and propidium iodide (PI) for 30 minutes in the dark. Cell cycle distribution was analyzed by BD FACSJazz flow cytometry (BD Biosciences).

Cell migration Assays

Cell migration assays were performed using 24-well Transwell chambers (Corning, Inc., Lowell, MA, USA) with a polycarbonate filter of 8 μm pore size. Briefly, approximately 5×10^4 transfected cells were seeded in RPMI-1640 medium without FBS into the upper chamber and 600 μl of serum-free medium was added to the lower chamber as a chemoattractant. After incubation for 16 hours, the cells in the upper chamber were removed with a cotton swab, and the migrated cells on the reverse side of the filter were fixed with 70% ethanol and stained with 0.1% crystal violet for 10 minutes. The number of migrating cells was counted in five randomly selected fields under the microscope (Olympus Corporation).

Wound Healing Assay

The transfected cells were plated in a 6-well plate. When the cells reached approximately 80% confluence, the monolayer of cells was scratched using a 200 μl pipette tip. The cell culture medium was then replaced with fresh medium. Wound healing was detected by phase contrast microscopy (Leica Microsystems Mannheim Germany) at 0 and 48 hours.

Xenografts in Mice

Briefly, BCa T24 cells with stable expression of si-LUM or si-NC were harvested, washed twice with PBS and resuspended in physiological saline solution. Approximately 5×10^6 cells were injected subcutaneously into the right neck of male BALB/C nude mice (age, 4–6 weeks old; weight, 18–22 g, five mice per group) that were purchased from Slaccas (Slaccas Laboratory Animal, Shanghai, China). The length and width of the tumor was measured every 3 days using a Vernier caliper, and the volume was calculated (Volume (mm^3) =

Table 1. The relationship between the expression of Lumican and various clinicopathological variables.

Characteristics	Total	Lumican expression		P value
		Low	High	
Total	97	37	60	
Age (years)				0.879
≤60	41	16	25	
>60	56	21	35	
Gender				0.424
Male	58	24	34	
Female	39	13	26	
Histological grade				**0.003****
Papilloma	14	6	8	
Low-grade carcinoma	44	24	20	
High-grade carcinoma	39	7	32	
T-stage				**0.013***
T1-T2	70	32	38	
T3-T4	27	5	22	
N-stage				**0.009****
N0	66	31	35	
N+	31	6	25	
M-stage				0.484
M0	72	26	46	
M1	25	11	14	

*$p < .05$, **$P < .01$.

0.5 × width^2 × length). Mice were treated humanely 4 weeks after the injection and the tumors were weighed. The animal study was conducted in accordance with the ethical guidelines for animal experiment systems approved by the Animal Care and Use Committee of Tongji University.

Statistical Analysis

Data were analyzed with GraphPad Prism software (Version 7.0, Inc., San Diego, CA, USA) and SPSS software (Version 20.0, SPSS, Inc., Chicago, IL, USA). The correlation of LUM expression with the patients' clinicopathological variables was analyzed using Fisher's exact test. A *P*-value <0.05 was considered statistically significant.

Results

LUM is Highly Expressed in BCa and Correlates with the Prognosis of BCa Patients

We analyzed the relationship between LUM expression and various clinicopathological variables in 97 patients (Table 1). The expression of LUM was found to be positively correlated with histological grade (P = .003), T-stage (P = .013) and N-stage (P = .009), but not related to age, gender and M-stage. IHC indicated that LUM was overexpressed in bladder tumor tissue compared with normal bladder tissue. Moreover, the expression of LUM in BCa tissue is higher than in papilloma tissues (Fig.1. *A–C*). Furthermore, Western blot experiments showed that the protein level of LUM in bladder tumor tissues and cell lines was higher than in normal bladder tissues and cells (Fig.1, *D* and *E*). In addition, BCa patients with higher LUM expression had a worse overall survival than patients with lower LUM expression (P = .021) when analyzed using the KM Plotter (http://kmplot.com/analysis/) (Fig.1*F*).

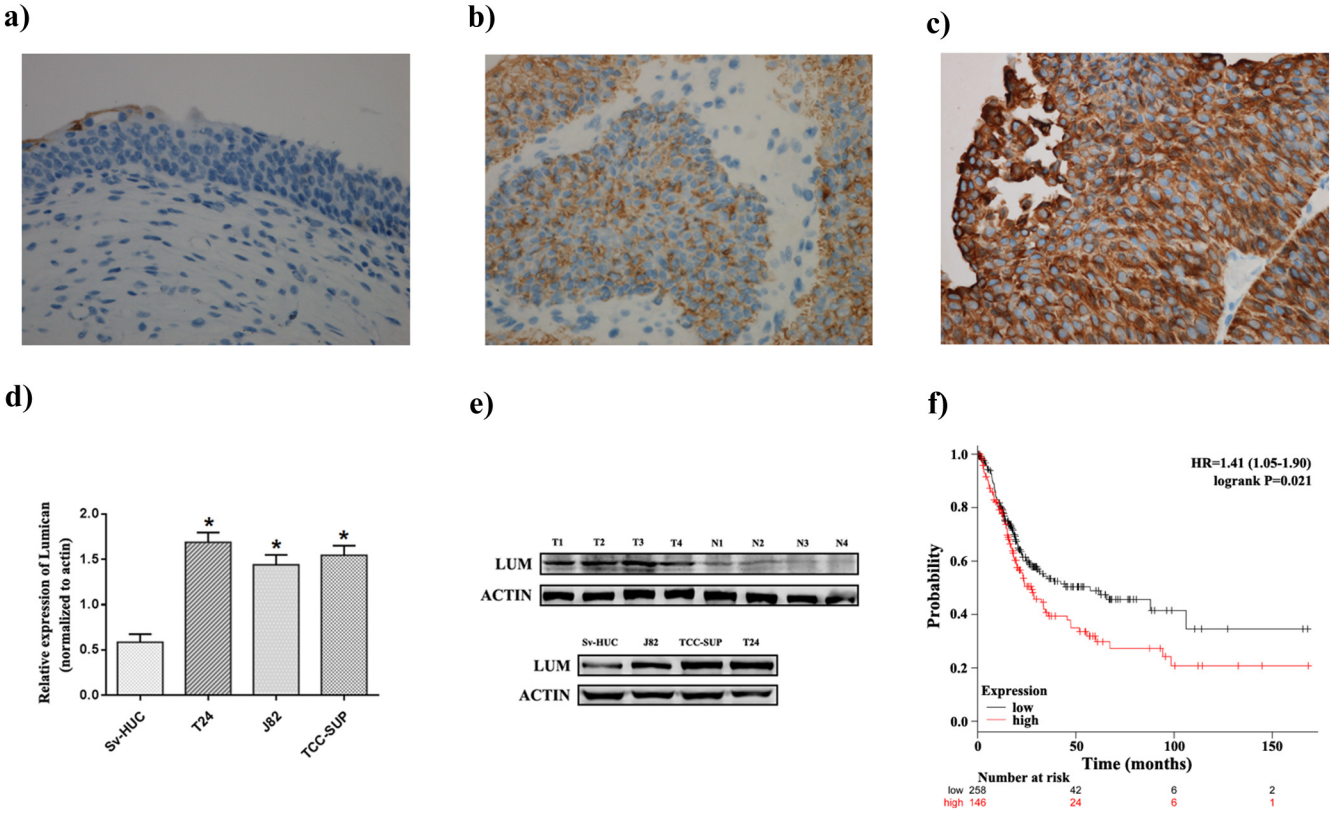

Fig. 1. LUM is highly expressed in BCa and correlated with prognosis of BCa patients. **a-c**, The expression of LUM in different histological grades; normal bladder tissue (**a**), papilloma tissue (**b**), and BCa tissue (**c**). **d** and **e**, Relative protein levels of LUM in BCa tissues (T) and adjacent normal tissues (N), BCa cell lines (T24, J82, and TCC-SUP) and normal bladder cell (Sv-HUC) (*$P < .05$). **f**, Kaplan–Meier plotter analysis of the correlation of LUM expression level with overall survival of BCa patients by the KM Plotter (P = .021).

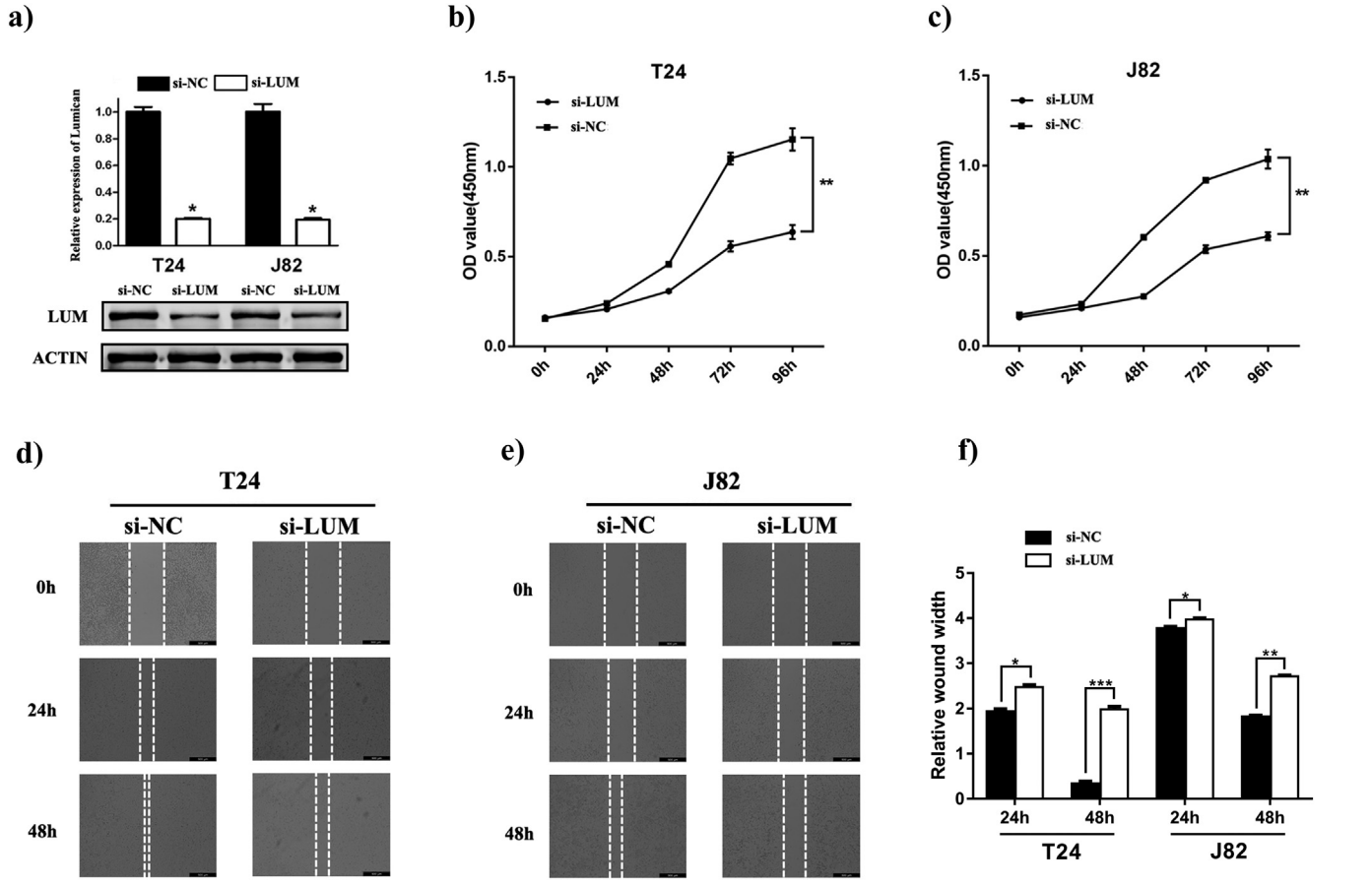

Fig. 2. Knockdown of LUM inhibited proliferation in BCa cells. **a**, Relative expression of LUM by qRT-PCR and Western bolt. **b** and **c**, Tumor cell growth was determined in T24 and J82 cell lines following transfection with si-NC or si-LUM (*$P < .05$). **d-f**, Wound healing assays were performed in T24 and J82 cells treated with si-NC or si-LUM (*$P < .05$).

Knockdown of LUM Inhibits Proliferation in BCa Cells

To investigate the role of LUM in BCa, we constructed stable cell lines with low LUM expression (si-LUM) and control cell lines (si-NC). The inhibition of LUM expression was confirmed by qRT-PCR and Western blot (Fig.2*A*). Functionally, the CCK-8 assay showed that knockdown of LUM significantly inhibited the proliferation of the BCa cell lines T24 and J82 (Fig.2, *B* and *C*). Furthermore, LUM knockdown cell lines also displayed defects in wound healing compared to si-NC cells (Fig.2, *D*–*F*).

Knockdown of LUM Decreases the Migration of BCa Cells and Induces Cell Cycle Arrest

To further evaluate the effect of LUM on BCa cells, we analyzed cell migration ability, cell cycle distribution and the expression of migration and cell cycle associated proteins in BCa cell lines transfected with si-NC or si-LUM. We found that the migration capacity was reduced and expression of the migration-associated protein MMP-2 was decreased after transfection of si-LUM in BCa cells in comparison to the control (Figure 3, *A*, *B* and *E*). In addition, a significant increase in the numbers of cells in the G0/G1 phase and a decreased number of cells in the S/M phase were observed in the si-LUM cells compared to the si-NC cells (Figure 3, *C* and *D*). Moreover, Western blot experiments showed that the cycle-related protein CyclinD1 was increased in the si-NC cell line compared to the si-LUM cell line (Fig.3*E*). In addition, we found that there was no significant change in the expression of ERK and MEK1/2 after knockdown of LUM, although the protein levels of p-EKR and p-MEK1/2 decreased significantly. These results suggest that the inhibition of LUM inactivated MAPK signal in BCa T24 and J82 cells.

Down-regulation of LUM Significantly Inhibits Cellular Growth In Vivo

To investigate the effect of LUM expression on BCa cell growth *in vivo*, we injected T24 cells stably transfected with sh-NC and sh-LUM into nude mice. The autopsy examination was performed after 27 days, and there we found that the volume and weight of tumors formed by sh-LUM transfected T24 cells was reduced compared to sh-NC transfected T24 cells (Fig.4a-c). Moreover, the expression of LUM in the tumors of the sh-LUM group was decreased by IHC (Fig.4d).

Discussion

SLRPs are a common matrix component, usually composed of a nucleoprotein and a covalently attached glycosaminoglycan chain [14,15]. It has been found that the SLRP family has 17 members, classified into five major classes (canonical classes I–III and noncanonical classes IV–V) based on the conserved nature of protein, N-terminal cysteine clusters, number of leucine-rich repeats sequences, type of glycosaminoglycan chain and gene structure [16,17].

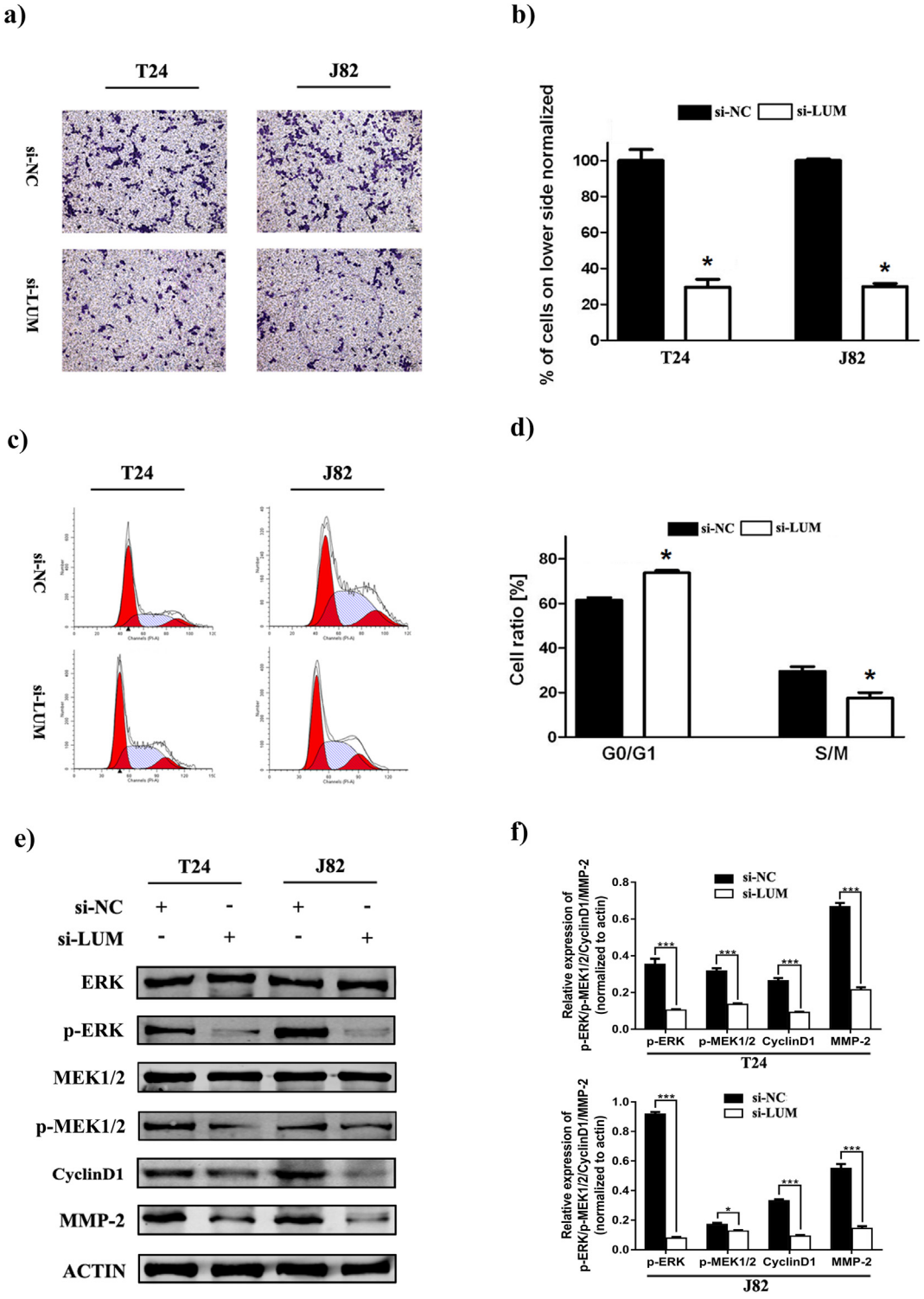

Fig.3. Knockdown of LUM induced migration decrease and cell cycle arrest in BCa cells. **a** and **b**, Cell migration assays were performed in EJ and UMUC3 cells using Transwell chambers (*$P < .05$). **c** and **d**, Cell cycle distribution of T24 and J82 after LUM knockdown as determined by flow cytometry (*$P < .05$). **e**, The protein expressions of ERK, p-ERK, MEK1/2, p-MEK1/2, MMP-2 and CyclinD1 were detected by Western blot in BCa cell lines T24 and J82 after knockdown of LUM. **f**, Relative protein expression of each protein in T24 and J82 cells was quantified using Image-J software and normalized to ACTIN.

LUM is a small-molecule proteoglycan with a relative molecular weight of approximately 38 kD and a members of the SLRP family type II [18]. As a secretory protein, LUM was first found to be expressed in the cornea and then in skeletal muscle, kidney, pancreas and intervertebral tissue [19,20]. As one of the constituent proteins of the extracellular matrix (ECM), LUM plays an important role in maintaining and regulating the microenvironment needed for tissue cell growth as well as cell proliferation, tissue self-stability,

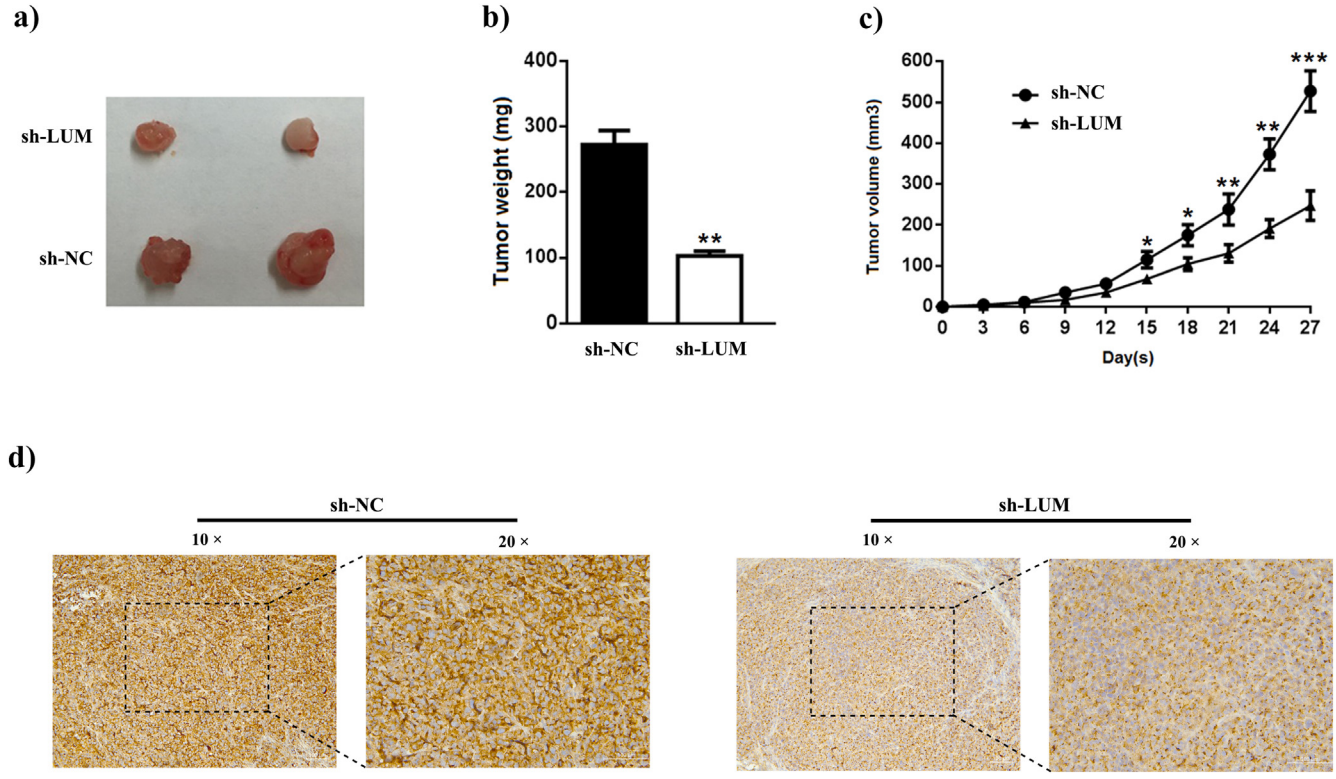

Figure 4. Downregulation of LUM significantly inhibited cellular growth *in vivo.* **a-c,** Tumor xenograft size, volume and weight in the sh-LUM and sh-NC groups. **d**, Immunohistochemistry (IHC) staining of LUM in xenografts. Scale bar = 100 μm for 10 × and 100 μm for 20 × .

inflammation, injury repair, epithelial-mesenchymaltransition (EMT) and other processes [21–25].

In recent years, an increasing amount of experimental data indicates that LUM is expressed in various types of tumor tissues [26–28]. Grzesiak et al. [29] found that LUM binds to integrins in pancreatic cancer tissue, thereby promoting tumor cell invasion and migration. Leygue et al. [30] found that LUM was the most expressed proteoglycan in breast cancer, and high expression of LUM was observed in tumor tissues and interstitium. Moreover, higher expression of LUM suggested worse prognosis in patients.

In a previous study, LUM has been reported to be highly expressed in BCa tissues [8]. However, the functional role of LUM in BCa is still unknown. In our study, we inhibited the expression of LUM by constructing si-LUM stable cell lines and found that knockdown of LUM could inhibit the proliferation, migration and cell cycle arrest of BCa cells. Moreover, the expression of the migration-associated protein MMP-2 and the cycle-related protein CyclinD1 was decreased. In addition, we also found that patients with higher LUM expression had a worse overall survival.

LUM regulates the growth and progression of BCa through different mechanisms [31,32]. Wang et al. [33] found that LUM is highly expressed in gastric cancer associated fibroblasts and promotes tumor growth by activating integrin β1 mediated FAK signaling pathway. Quan et al. [34] found that silencing LUM can reduce the invasiveness of colon cancer SW480 and HCT-8 cells through MAPK signaling pathway. In terms of mechanism, by knocking down the expression of LUM, we found that there was no significant change in the expression of ERK and MEK1/2, but the protein levels of p-EKR and p-MEK1/2 decreased significantly. The results showed that LUM silencing could inhibit the progress of BCa by inactivating MAPK signal transduction.

Conclusions

In conclusion, current results indicate that LUM is highly expressed in BCa tissues and cell lines and that increased LUM expression is associated with the histological grade and the T/N stage of bladder tumors. The *in vitro* and *in vivo* data further indicate that low expression of LUM can inhibit the growth and migration of BCa cells by inactivating MAPK signaling. Therefore, our study suggests that LUM may serve as a novel target for the treatment of BCa.

Conflicts of Interest

The authors of this manuscript have no conflict of interest.

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Author Contribution

WM, YZ and JG designed the research. ML, XH, QW and JF acquired the data. WM, LG and YZ analyzed the results. WM wrote the article. YZ and JG revised and provided critical comments. All authors read and approved the final manuscript.

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