

Downregulation of *LAPTM5* suppresses cell proliferation and viability inducing cell cycle arrest at G0/G1 phase of bladder cancer cells

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Abstract. Our transcriptome analysis revealed in bladder cancer (BCa) tissues a significant induction of lysosomal-associated multispanning membrane protein 5 (*LAPTM5*), a lysosomal membrane protein preferentially expressing in immune cells and hematopoietic cells. Transportation of *LAPTM5* from Golgi to lysosome could be inhibited by deficiency of Nedd4, a key member of E3 ubiquitin ligase family overexpressing in invasive BCa and promoting its progression. Therefore, we hypothesize that *LAPTM5* may be closely correlated with BCa tumorigenesis. In human BCa tissues, we observed that *LAPTM5* was significantly induced at both mRNA and protein levels, which is consistent with our microarray result. Furthermore, we established a BCa cell model with downregulated *LAPTM5*, revealing a significantly delayed growth rate in the BCa cells with knockdown of *LAPTM5*. Moreover, cell cycle arrest at G0/G1 phase was triggered by decreased *LAPTM5* as well, which could lead to delayed BCa cell growth. In contrast, no significant alteration of apoptosis in the BCa cells with downregulated *LAPTM5* was noticed. Analysis of the changes of migration and invasion, showed significant reduced *LAPTM5* suppressed cell metastasis. Furthermore, proteins involved in epithelial-mesenchymal transition (EMT) were strongly altered, which

plays a central role in metastasis. In addition, phosphorylated ERK1/2 and p38, key members of mitogen-activated protein kinase (MAPK) family regulating BCa tumorigenesis, were strongly decreased. Taken together, our results suggested that decreased *LAPTM5* inhibited proliferation and viability, as well as induced G0/G1 cell cycle arrest possibly via deactivation of ERK1/2 and p38 in BCa cells.

Introduction

Human bladder cancer (BCa) is currently one of the most common cancers worldwide (1). However, after complex therapies including surgery and antineoplastic therapy, BCa still frequently recurs and eventually progresses into muscle-invasive BCa (2). Therefore, new specific molecular markers and effective therapies are urgently needed.

Our group has collected several human BCa tissues and normal bladder tissues to conduct a microarray analysis (GEO accession no. GSE76211) (3,4), revealing a significantly upregulated gene in BCa tissues, the lysosomal-associated protein multispanning transmembrane 5 (*LAPTM5*). *LAPTM5* is a lysosomal membrane protein preferentially expressed in immune cells (5,6) and hematopoietic cells (7), having a close interaction with the Nedd4 (8), a member of the E3 ubiquitin ligases family (8). Nedd4 has been shown to be specifically upregulated in invasive BCa and be able to promote the progression of BCa (9). Moreover, some studies demonstrated that *LAPTM5* was highly expressed in malignant B lymphomas and involved in B cell malignancies (10), involving in negative regulation of cell surface T and B cell receptor by promoting lysosome degradation (6). Furthermore, previous studies suggested that knockdown of *LAPTM4B*, another important subtype of the *LAPTM* family inhibited proliferation of hepatocellular carcinoma (11), prostate (12) and breast cancer cells (13).

In recent years, epithelial-mesenchymal transition (EMT) has been suggested to play a key role in the process of embryonic development, differentiation of tissues and organs, chronic inflammation and fibrosis, as well as cancer progres-

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sion (14). During EMT, cells will undergo transformation from epithelial phenotype to mesenchymal phenotype (14) and many characteristics of cells will change including loss of cell-cell adhesion and acquisition of aggressive and metastatic ability (15). Increasing evidence suggested EMT was involved in cancer invasion, metastasis (16) and the malignancy of tumors (17), often marked by reduction of E-cadherin and induction of N-cadherin (18). However, whether LAPTM5 has a connection with EMT in BCa cells remains largely unknown.

Our transcriptome analysis suggested that mitogen-activated protein kinase (MAPK) signaling pathway was linked with bladder cancer by participating in cell cycle regulation (3,4). In addition, recent studies reported that LAPTM5 could diminish the activation of MAPK signaling pathway regulated by tumor necrosis factor (TNF) receptor (19). More importantly, abnormal regulation of MAPK could contribute to cancer and other human diseases (20,21), including bladder cancer (3).

The exact role of LAPTM5 in tumorigenesis of human bladder cancer has not been investigated previously. In the present study, we first demonstrated that reduction of *LAPTM5* had negative effects on migration, invasion and proliferation of BCa cells. Furthermore, our results suggested that alteration of MAPK signaling pathway might participate in regulation of these processes.

Materials and methods

Ethical statement for human bladder samples. As described by Cao *et al* and Wang *et al* in 2016 from our group (3,4), bladder cancer and paracancerous tissue samples (n=13) were obtained from patients after surgery at Zhongnan Hospital of Wuhan University, and normal bladder tissue samples (n=3) were from donors by accidental death. The histology diagnosis was confirmed pathologically by two pathologists independently. All the tissues were immediately frozen and stored in liquid nitrogen or fixed in 4% PFA after collection from the operation room. Informed consent was collected from all subjects. The study using human bladder tissue samples for RNA isolation and immunohistochemistry staining analysis was approved by the Ethics Committee at Zhongnan Hospital of Wuhan University (approval no. 2015029). All methods used for human bladder tissue samples were performed in accordance with the approved guidelines and regulations.

Human bladder cancer cell lines. The human BCa cell lines T24 (transitional cell carcinoma, cat. no. SCSP-536) and 5637 (grade II carcinoma, cat. no. TCHu 1) were obtained from Chinese Academy of Sciences in Shanghai. T24 and 5637 cell lines were identified by the China Centre for Type Culture Collection in Wuhan, China. T24 and 5637 were cultured in RPMI-1640 medium (Gibco, China) containing 10% fetal bovine serum (FBS) (Gibco, Sydney, Australia) in a humidified atmosphere with 5% CO₂ at 37°C.

RNA expression analyses

Total RNA isolation from bladder tissues and BCa cells. Total RNA was isolated from BCa cells and bladder tissues by RNeasy mini kit (cat. no. 74101), combined with QIAshredder (cat. no. 79654) (both from Qiagen, Hilden, Germany) using

a centrifuge (cat. no. 5424; Eppendorf, Hamburg, Germany), according to the manufacturer's protocol. In order to remove genomic DNA, DNase I digestion (cat. no. 79254; Qiagen) was used in each RNA preparation.

Reverse transcription and quantitative real-time PCR (qRT-PCR). For each sample, First-Strand cDNA was synthesized using 1 µg of total RNA isolated from BCa cells or bladder tissues by ReverTra Ace qPCR RT kit (Toyobo, Shanghai, China). Each reaction was conducted with iQ™ SYBR®-Green Supermix (Bio-Rad, Shanghai, China) using 1 µg of cDNA in a final volume of 20 µl. All primers were tested for optimal annealing temperatures and PCR conditions were optimized with gradient PCRs on an iCycler (cat. no. CFX Connect; Bio-Rad, Hercules, CA, USA). Primer sequences and annealing temperatures are summarized in Table I. The cycle number of threshold (CT) value of *LAPTM5* was normalized to the *GAPDH* value, and calculated as (22): relative gene expression = $2^{-\Delta\Delta Ct}$, $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{GAPDH}}$, for BCa cells $\Delta\Delta Ct = \Delta Ct_{\text{siRNA-treated}} - \Delta Ct_{\text{siRNA-untreated}}$, for bladder tissues $\Delta\Delta Ct = \Delta Ct_{\text{BCa tissues}} - \Delta Ct_{\text{paracancerous tissues}}$ (ct, threshold cycle).

Cell culture analyses

Knockdown of LAPTM5 in the BCa cells. *LAPTM5*-target specific small interfering RNA (siRNA) was synthesized by View Solid (Beijing, China). The sense sequence of *LAPTM5*-target-specific-siRNA (*si-LAPTM5*) is as follows: siRNA1, 5'-CCACCUAUCUCAACUUAATT-3'; siRNA2, 5'-CCAU CUACCAUGUGAUCAUTT-3'; siRNA3, 5'-GGUGCUACAG AUUGAUCAATT-3', and the sense sequence of *si-control* is 5'-UUCUCCGAACGUGUCAGGUTT-3'. When cells were grown to 60%, T24 and 5637 cells were transfected with *si-LAPTM5* and *si-control* using LipoJet™ (SignaGen, China). After 48 h transfection, alterations of *LAPTM5* mRNA and protein were evaluated by qRT-PCR, immunofluorescence staining and western blot analyses.

Transwell chamber migration and invasion assay. The Transwell migration and invasion assay was conducted in 24-well plate Transwell chamber system (Corning, Inc., NY, USA). For the migration, BCa cells in serum-free medium at a density of $4-6 \times 10^4$ cells were seeded in the upper chamber (Corning, Inc.), while the lower chamber was filled with 10% FBS medium. After incubation for 24 h at 37°C, the cells were removed using cotton swabs in the upper chamber. Then lower side of the chamber was fixed with 4% PFA and stained with crystal violet, migrated cell number was counted by phase contrast microscope and statistically analyzed. To perform invasion assay, Transwell chambers were percolated with ECM Matrix gel solution (Sigma-Aldrich, St. Louis, MO, USA). Then solidified at 37°C, $\sim 1 \times 10^5$ cells were seeded as previously described. The chamber was incubated at 37°C for 48 h. The subsequent staining and observation procedures were identical to those of the migration assays.

Wound healing assay. After siRNA-transfection for 24 h, BCa cells were scratched, and washed with PBS. Adding 0.5% FBS medium to allow cells to move into the gap, they were photographed at 0 and 12 h in several pre-marked spots. Migration rate was statistically analyzed using t-test.

MTT assay. After transfection for 48 h, 3,000-5,000 BCa cells/200 µl medium were seeded in 96-well plates to grow for another four days. Then 20 µl MTT was added in each well and

Table I. The primers for qRT-PCR.

Gene	Symbol	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature (°C)	Length (bp)
Lysosomal-associated multispanning membrane protein 5	<i>LAPTM5</i>	5'-CCTGAGCCTA CTGATCGGC-3'	5'-CAGGCACAGGA GATAGTCCA-3'	60	91
Glyceraldehyde-3-phosphate dehydrogenase	<i>GAPDH</i>	5'-TGCACCACCAA CTGCTTAG-3'	5'-GATGCAGGGA TGATGTTC-3'	60	176

incubated at 37°C for 4 h. After removing the medium, formazan precipitate was dissolved in DMSO, and absorbance at 490 nm was measured by a microplate reader (cat. no. SpectraMax M2; Molecular Devices, Sunnyvale, CA, USA).

Clonogenic survival assay. BCa 1,000-1,500 cells/well were seeded in new 6-well plates and grew into colonies for ~15 days. Colonies were emerged and fixed by 4% PFA for 30 min, stained with 0.1% crystal violet for observation and photographing.

Flow cytometry analysis for cell cycle arrest and apoptosis. After harvesting and washing by PBS, BCa cells were fixed with 70% ice cold ethanol (-20°C, overnight), washed again and incubated with RNaseA (20 µg/ml in PBS), stained by propidium iodide (50 µg/ml) for 30 min (Sigma-Aldrich) at 37°C in the dark. Cell cycle were assessed on a flow cytometry (cat. no. FC500; Beckman Coulter, USA). Cell apoptosis analysis was analyzed by the flow cytometry analysis using Annexin V-fluorescence isothiocyanate (FITC)/PI apoptosis detection kit (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions.

Protein analyses

Western blot analyses. Total protein of BCa cells was extracted using RIPA buffer containing protease inhibitor and phosphatase inhibitor (Sigma-Aldrich). Bradford protein assay (Bio-Rad, Munich, Germany) was used to measure protein concentration and Bovine serum albumin (BSA) as a standard. Protein samples were separated using 10-12.5% SDS-PAGE and transferred to PVDF membrane (Millipore, Billerica, MA, USA). PVDF membranes were blocked in 5% non-fat milk, then incubated with primary antibodies (Table II) and secondary antibodies (Table III). Bands were visualized and blots were exposed to Kodak Biomax MR film after using an enhanced chemiluminescence (ECL) kit (Bio-Rad).

Immunofluorescence staining for BCa cells. Coverslips were washed 3 times by cold PBS and fixed with 4% PFA for 30 min. Then the cells were treated by 0.1% Triton X-100 and blocked in goat serum for 30 min, incubating with primary antibody (Table II) at room temperature for 2 h, washing with PBS and incubating with Cy3-labeled or FITC-labeled secondary antibody (Table III) for 1 h. Nuclei were labeled with DAPI (2 µg/ml). Immunofluorescence staining was analyzed using a fluorescence microscope (cat. no. IX73; Olympus, Japan).

Immunohistochemistry (IHC) staining for BCa tissue samples. Briefly, tissues were incubated with citrate buffer (0.01 M, pH 6.0) for 10 min after hydrated and embedded. After washing with PBS (pH 7.4) three times, tissue sections were covered with 3% H₂O₂ for 15 min at room temperature and incubated with primary antibody overnight at 4°C. After a washing procedure, biotinylated secondary antibody was incubated with the section for 30 min. Then DAB substrate chromogen solution was added before tissue sections were incubated with HRP substrate solution for 30 min. Slides were counterstained for 1 min with hematoxylin, then dehydrated and analyzed by microscopy.

Statistical analyses. All analyses were performed three times and represent data from three individual experiments. Two-tailed Student's t-test was used for significance of differences between subgroups. Statistical analyses were performed with SPSS 16.0. Statistical significance was set at probability values of p<0.05.

Results

Upregulation of LAPTM5 in BCa tissues compared with paracancerous tissues and normal bladder tissues. Oncomine database (www.oncomine.org) showed that *LAPTM5* was significantly upregulated at the transcriptional level in BCa tissues compared with normal bladder tissues (Fig. 1A), which is consistent with our microarray data. Furthermore, *LAPTM5* also exhibited a significant upregulation in the BCa tissues compared with the paired paracancerous tissues (n=13) (Fig. 1B). In addition, immunohistochemistry staining revealed strong increase of LAPTM5 protein in the BCa tissues, compared with paracancerous bladder tissues (Fig. 1C).

Knockdown of LAPTM5 significantly inhibits the transcription and protein levels of LAPTM5. To construct a cell model of *LAPTM5* deficiency, we used three distinct *LAPTM5*-target-specific-siRNA to transfect T24 and 5637. After 48 h, the knockdown efficiency was validated by qRT-PCR (Fig. 2A and B) and western blot analysis (Fig. 2C). Moreover, immunofluorescence staining also showed the abundance of LAPTM5 protein was strongly downregulated (Fig. 2D). The result showed that LAPTM5 expression at both transcriptional and translational levels was significantly reduced with *LAPTM5*-target-specific-siRNA in the BCa cells.

Table II. The primary antibodies.

Antigens	Species antibodies raised in	Dilution (IF)	Dilution (WB)	Supplier
E-Cadherin, human	Rabbit, monoclonal	1:200	1:500	Cell Signaling Technology, USA, cat. no. 3195
N-Cadherin, human	Rabbit, monoclonal	1:200	1:1,000	Cell Signaling Technology, USA, cat. no. 13116
β -Catenin, human	Rabbit, monoclonal	-	1:1,000	Cell Signaling Technology, USA, cat. no. 8480
Ki-67, human	Rabbit, monoclonal	1:200	-	Novus Biologicals, USA, cat. no. NBP2-19012
Slug, human	Rabbit, monoclonal	-	1:1,000	Cell Signaling Technology, USA, cat. no. 9585
Claudin-1, human	Rabbit, monoclonal	-	1:10,000	Cell Signaling Technology, USA, cat. no. 13255
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), human	Mouse, monoclonal	-	1:2,000	Santa Cruz Biotechnology Inc., USA, cat. no. sc-365062
Cyclin D1, human	Rabbit, monoclonal	-	1:1,000	Cell Signaling Technology, USA, cat. no. 2978
CDK2, human	Rabbit, monoclonal	-	1:1,000	Cell Signaling Technology, USA, cat. no. 2546
CDK4, human	Rabbit, monoclonal	-	1:1,000	Abcam, UK, cat. no. ab108357
Cyclin A1/A2	Rabbit, monoclonal	-	1:1,000	Abcam, UK, cat. no. ab185619
p-GSK3 β , human	Rabbit, monoclonal	-	1:10,000	Cell Signaling Technology, USA, cat. no. 5558S
GSK3 β , human	Rabbit, monoclonal	-	1:10,000	Cell Signaling Technology, USA, cat. no. 12456S
LAPTM5, human	Rabbit, monoclonal	1:50	1:1,000	Abcam, UK, cat. no. ab108014
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), human	Rabbit, monoclonal	-	1:1,000	Cell Signaling Technology, USA, cat. no. 4370
p44/42 MAPK (Erk1/2), rat	Rabbit, monoclonal	-	1:1,000	Cell Signaling Technology, USA, cat. no. 4695
Phospho-p38 (Thr180/Tyr182), human	Rabbit, monoclonal	-	1:1,000	Cell Signaling Technology, USA, cat. no. 4511
p38 MAPK, human	Rabbit, monoclonal	-	1:1,000	Cell Signaling Technology, USA, cat. no. 8690

Table III. The secondary antibodies and counterstaining of nuclei.

Secondary detection system used	Host	Method	Dilution	Supplier
Anti-mouse-IgG (H+L)-HRP	Goat	WB	1:10,000	Sungene Biotech, China, cat. no. LK2003
Anti-rabbit-IgG (H+L)-HRP	Goat	WB	1:5,000	Sungene Biotech, China, cat. no. LK2001
Anti-rabbit IgG (H+L), F(ab') ₂ fragment (Alexa Fluor [®] 488 Conjugate)	Goat	IF	1:50	Cell Signaling Technology, USA, cat. no. 4412
Hoechst 33342 (1 mg/ml) nucleic acid staining (DAPI)	-	IF	1:750	Molecular Probes/Invitrogen, Carlsbad, CA, USA, cat. no. A11007

Downregulation of LAPTM5 restrains proliferation of BCa cells. To detect the effect of *LAPTM5* knockdown on cell viability in BCa cells, T24 and 5637 were treated by *LAPTM5-siRNA* and *si-control* for 48 h and determined

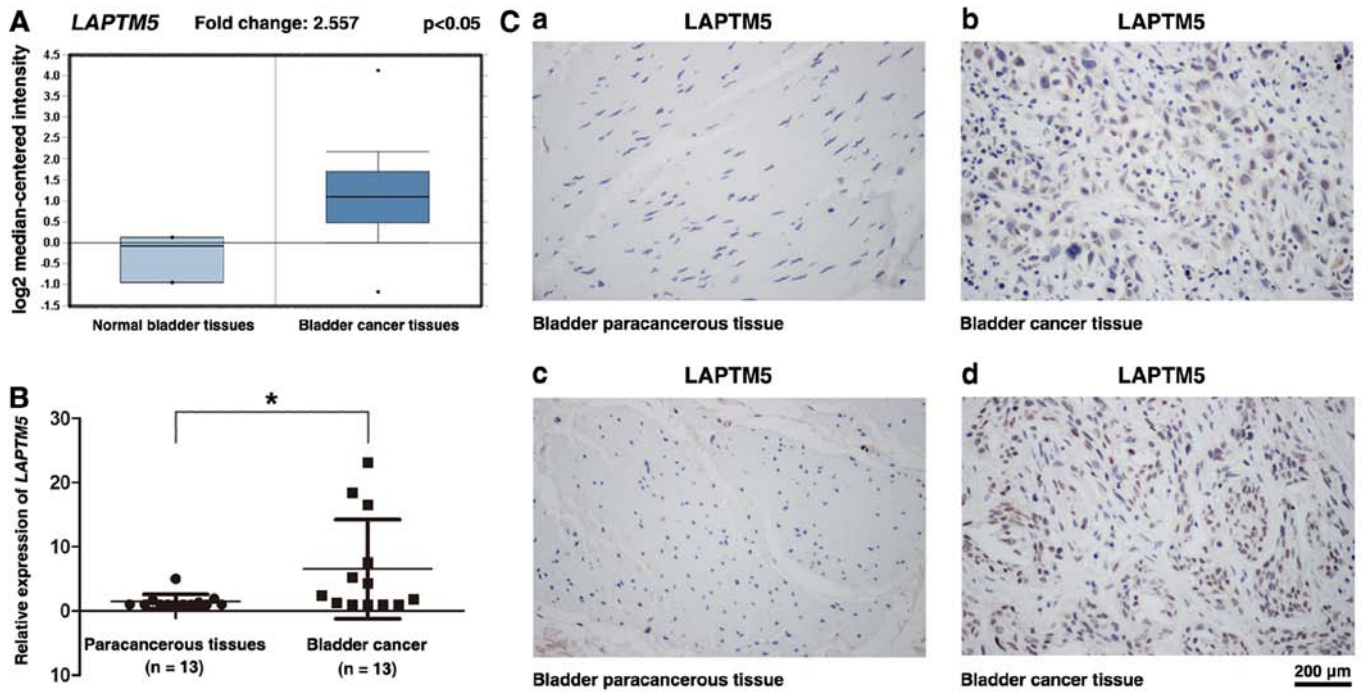


Figure 1. *LAPT5* is strongly upregulated in BCa tissues compared with paracancerous tissues and normal bladder tissues. (A) Upregulation of *LAPT5* mRNA expression in bladder cancer analyzed by Oncomine database. Analysis using the Oncomine database (www.oncomine.org) revealed increased *LAPT5* at transcriptional level in bladder cancer versus normal bladder tissues. (B) qRT-PCR analysis showed that the gene expression of *LAPT5* in bladder cancer tissues was significantly higher than the matched paracancerous tissues. The *GAPDH* value was used as an internal control. * $p < 0.05$. (C) Representative immunohistochemistry staining of *LAPT5* (brown) in (b and d) bladder cancer tissues and (a and c) paracancerous tissues. The scale bar for (C) is 200 μ m.

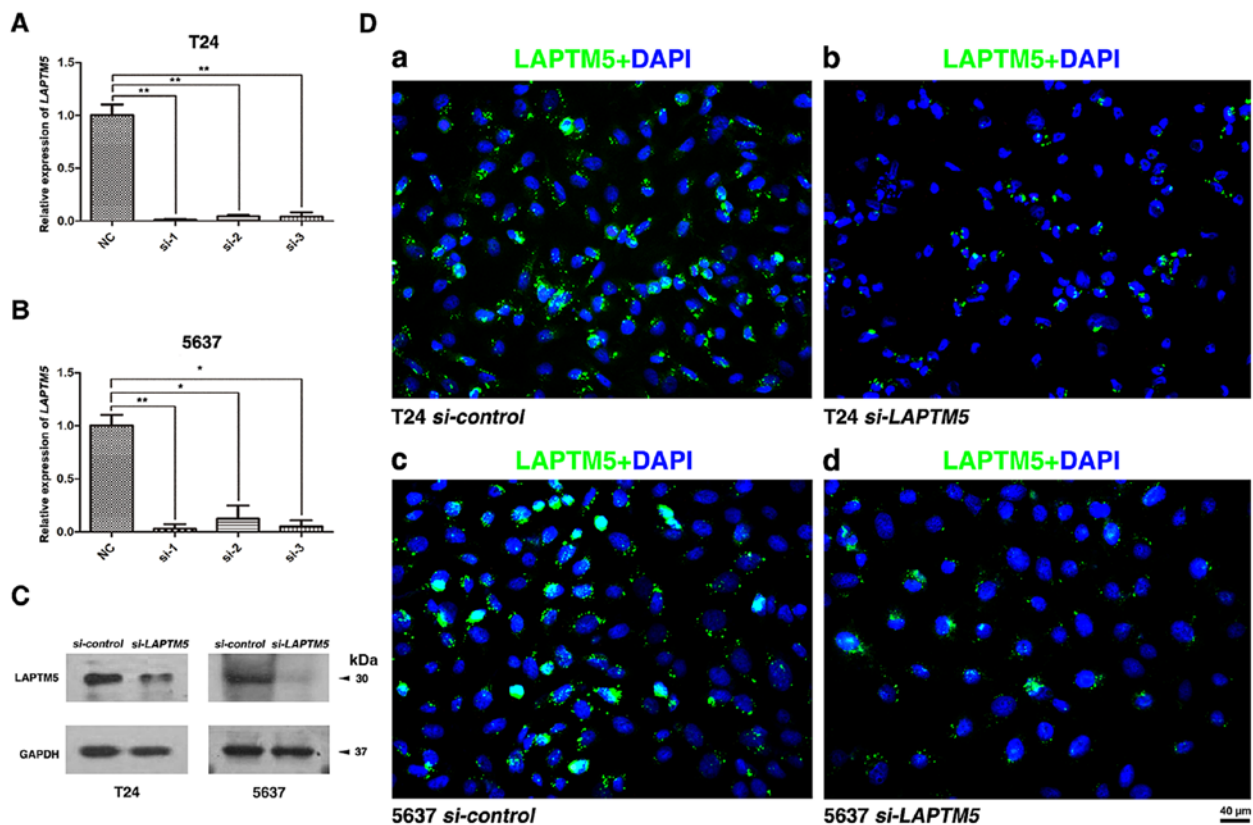


Figure 2. Establishment of BCa cell model with downregulated *LAPT5*. (A and B) qRT-PCR validated the efficiency by using distinct *siRNA* to knockdown *LAPT5* at transcriptional level in the BCa cells T24 and 5637. All values shown are mean \pm SD of triplicate measurements and repeated three times with similar results, * $p < 0.05$ and ** $p < 0.01$. (C) Western blot analysis revealed a strongly decreased protein abundance of *LAPT5* by the *LAPT5*-target-specific-*siRNA* (*si-LAPT5*) treatment, comparing with control-*siRNA* (*si-control*) treatment. *GAPDH* was used as a loading control. (D) Representative immunofluorescence staining of *LAPT5* (green) in the BCa cells after *si-LAPT5* (b and d), compared with *si-control* (a and c) treatment. Nuclei were stained by DAPI (blue). The scale bar for (D) is 40 μ m.

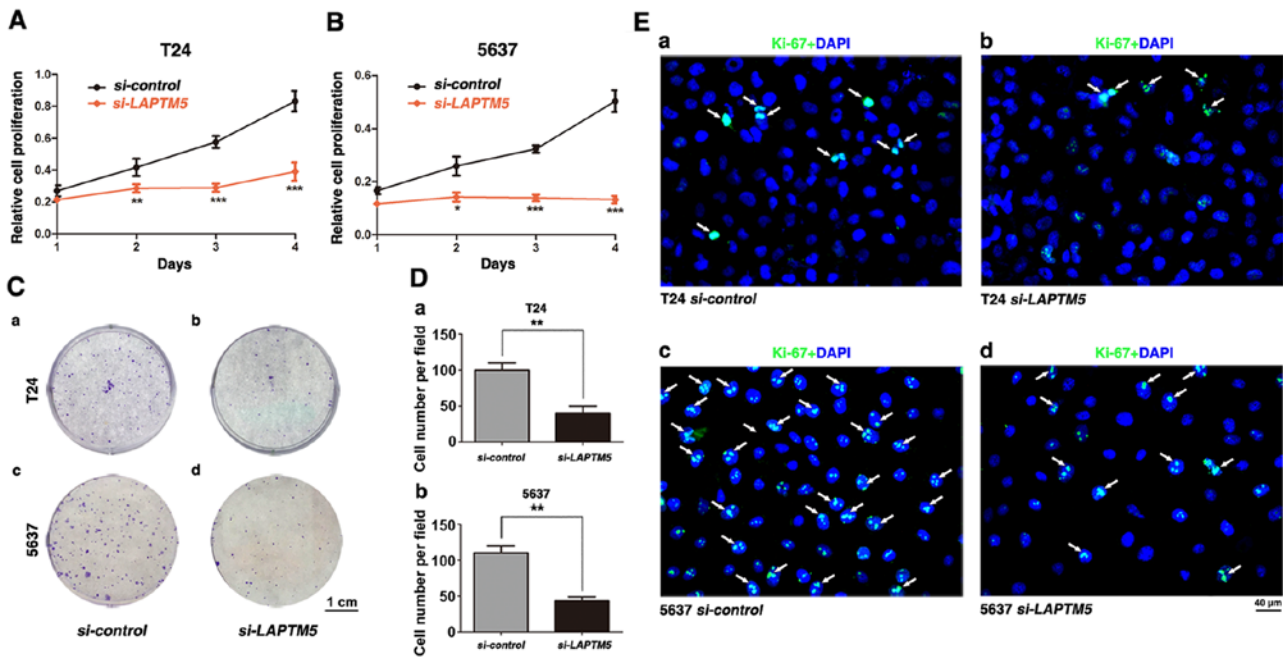


Figure 3. Downregulation of *LPTM5* represses BCa cell proliferation. (A and B) MTT assay was used to detect the viability of the two BCa cells treated by control-siRNA (*si-control*, black line) and *LPTM5*-target-specific-siRNA (*si-LPTM5*, red line). All values shown are mean \pm SD of triplicate measurements and repeated three times with similar results, ** $p < 0.01$ and *** $p < 0.001$. (C) Alteration of cell survival for T24 (a and b) and 5637 (c and d) were analyzed by clonogenic survival assay after treatment with *si-control* and *si-LPTM5*. (D) Clone number in each well was counted and statistically analyzed in the clonogenic survival assay. ** $p < 0.01$. (E) Cell proliferation of BCa cells treated by *si-control* (a and c) and *si-LPTM5* (b and d) was detected by Ki-67 staining (green), pointed by the arrows. Nuclei were stained by DAPI (blue). The scale bar for (C) is 1 cm and for (E) is 40 μ m.

by MTT assay, suggesting that knockdown of *LPTM5* restrained BCa cells proliferation drastically (Fig. 3A and B). Clonogenic survival assay revealed a significant reduction for the colony forming efficiency in the *LPTM5*-siRNA-treated BCa cells T24 and 5637, compared with the *si-control* group (Fig. 3C and D). Moreover, immunofluorescence staining showed that the *LPTM5*-siRNA group exhibited considerably less Ki-67 positive cells than the *si-control* group (Fig. 3E).

Reduced LPTM5 triggers cell cycle arrest at G0/G1 phase, but shows no significant changes on apoptosis in the BCa cells. Flow cytometry analysis was conducted to evaluate the effect of *LPTM5* knockdown on cell cycle in T24 and 5637 cells (Fig. 4A), indicating a significant cell cycle arrest at G0/G1 phase (Fig. 4B). Western blot analysis revealed that proteins involved in G0/G1 phase regulation were strongly reduced (cyclin A1/2, cyclin D1 and CDK2/4) after *LPTM5*-siRNA treatment (Fig. 4C). However, knockdown of *LPTM5* could not affect apoptosis in BCa cells significantly (Fig. 4D and E), as revealed by flow cytometry analysis.

Downregulation of LPTM5 inhibits migration and invasion of BCa cells. Transwell migration and invasion assay suggested that knockdown of *LPTM5* in BCa cells could reduce cell migration and invasion (Fig. 5A), which was confirmed by statistical analysis in Fig. 5B. Moreover, wound healing assay revealed that reduction of *LPTM5* in BCa cells could suppress the number of migrated cells (Fig. 5A). The gap closure (%) was statistically analyzed (Fig. 5B).

Proteins involved in MAPK signaling pathway and EMT regulation are altered after LPTM5 knockdown. Key members of

the MAPK family including ERK1/2 and p38 were affected in the *si-LPTM5*-treated T24 and 5637 cells (Fig. 5C). *LPTM5* knockdown strongly suppressed the expression of phosphorylated ERK1/2 (p-ERK1/2) and phosphorylated p38 (p-p38) in the BCa cells. In addition, proteins involved in the EMT process, including β -catenin, N-cadherin, E-cadherin, claudin-1 and Slug, were analyzed by western blot analysis (Fig. 5D), showing that the epithelial marker E-cadherin was upregulated and mesenchymal marker N-cadherin, β -catenin, Slug, claudin-1 were downregulated after *LPTM5* knockdown.

Discussion

Our group has established a transcriptome analysis using bladder cancer tissues versus normal bladder tissues (3,4). Among thousands of strongly altered genes involved in development of human bladder cancer (BCa), we selected the upregulated gene *LPTM5*, which is in accordance with the result from the Oncomine database. *LPTM5* has been reported to be correlated with NEDD4 (8) which is upregulated in invasive BCa and could promote progression of BCa (9). Interestingly, our results showed that the expression of *LPTM5* was strongly enhanced in BCa tissues at both transcriptional and protein levels compared with paraneoplastic tissues. The *LPTM5*-siRNA was used for *LPTM5* knockdown and the efficiency was confirmed by qRT-PCR, western blot analysis and immunofluorescence staining analyses. We observed that knockdown of *LPTM5* could reverse the EMT status, suggesting that deficiency of *LPTM5* could alleviate malignancy of BCa. Since several studies have reported that EMT was involved in cancer cell migration and invasion (16), we also observed that knockdown of *LPTM5*

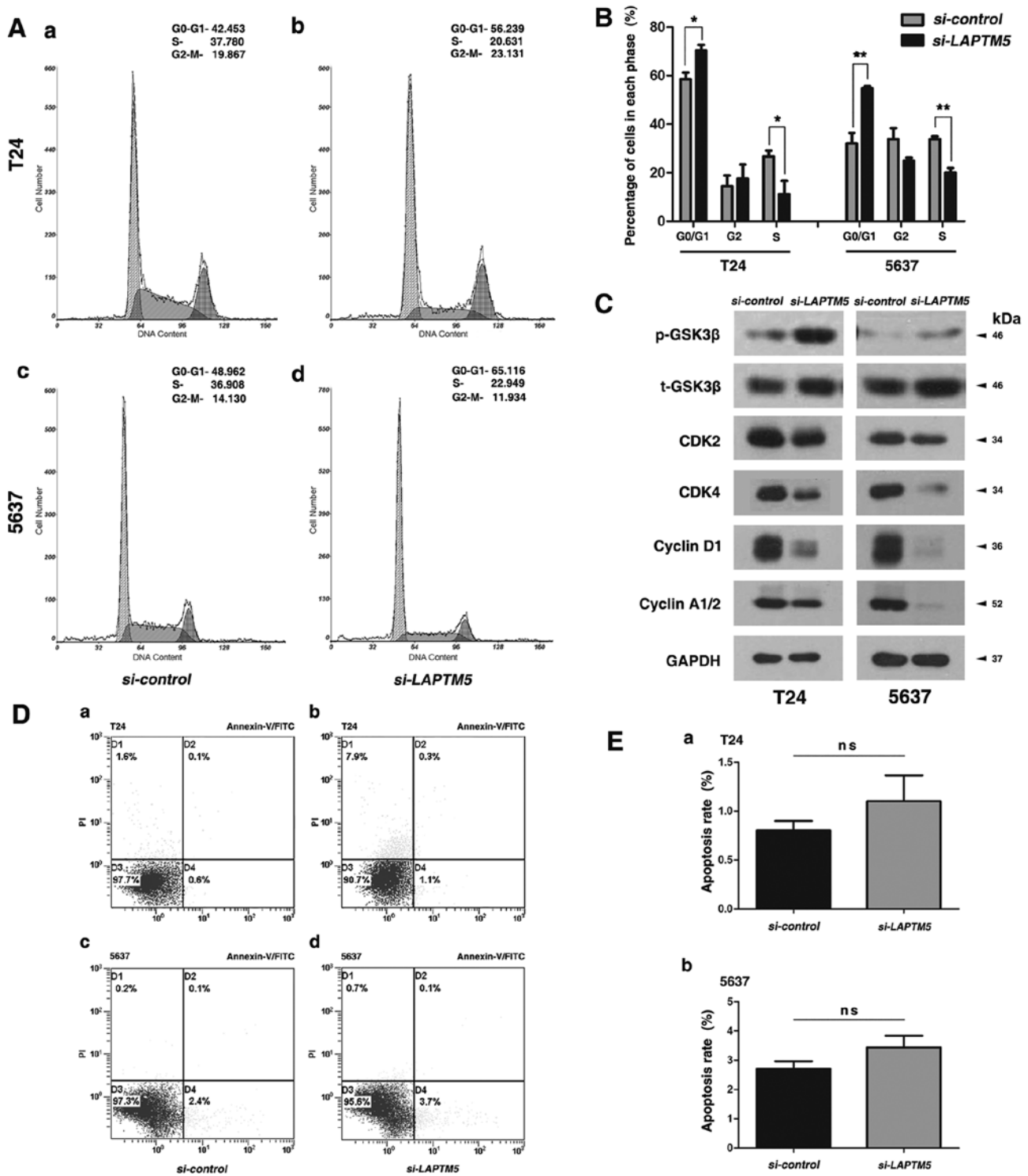


Figure 4. Knockdown of *LAPTM5* induces cell cycle arrest at G0/G1 phase in BCa cells. (A) Flow cytometry analysis for T24 (a and b) and 5637 cells (c and d) treated with *control-siRNA* (*si-control*) (a and c) and *LAPTM5-target-specific-siRNA* (*si-LAPTM5*) (b and d) for 48 h. (B) Alteration of percentages (%) of cell populations at different stages of cell cycles in T24 and 5637 cells was statistically analyzed. All the values shown are mean \pm SD of triplicate measurements and repeated three times with similar results. * $p < 0.05$ and ** $p < 0.01$. (C) Western blot analysis of proteins involved in the G0/G1 cell cycle regulation (cyclin A1/2, cyclin D1 and CDK2/4) and phosphorylated/total GSK-3 β in the BCa cells. GAPDH abundance was used as a loading control. (D) Flow cytometry analysis of alterations of BCa cell apoptosis (T24 and 5637) by the transfection using *si-control* and *si-LAPTM5*. Calculation area of the apoptosis rate was percentage of Annexin V/PI⁺ cells. (E) Statistical analysis suggested no significant (ns) induction of apoptosis by the downregulation of *LAPTM5* in human T24 (a) and 5637 (b) BCa cells.

suppressed migration and invasion of BCa cells. E-cadherin was found to be associated with epithelial cell migration and could play a key role in EMT progression (23), often marked by decreased E-cadherin and increased N-cadherin (18).

After re-localization from membrane to cytoplasm and nucleus, β -catenin became a transcriptional coactivator to promote EMT (24). Slug is a zinc-finger transcription factor and has a functional role in triggering EMT (25), cancer

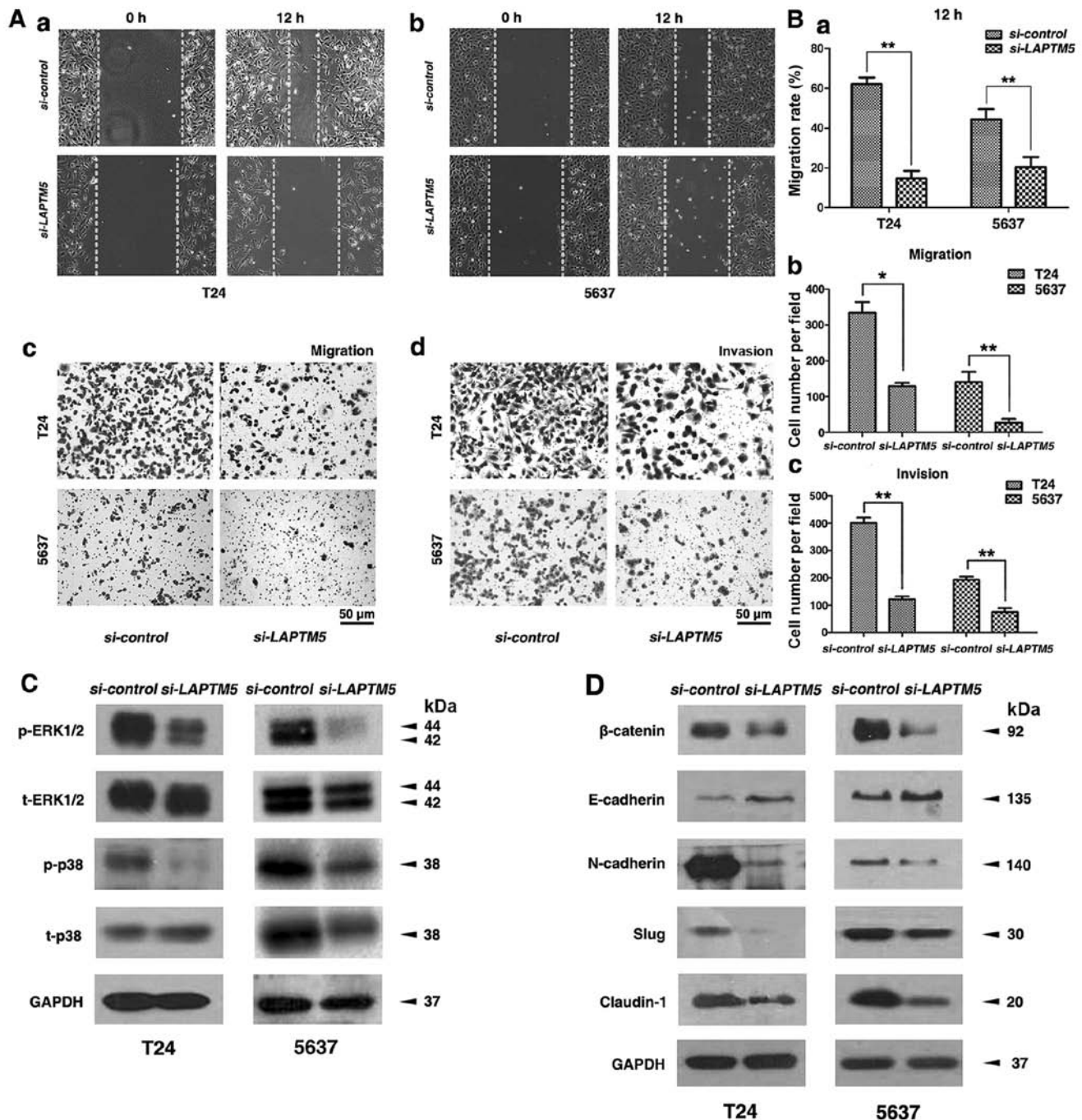


Figure 5. Proteins involved in MAPK signaling pathway and EMT regulation are altered after LAPTM5 knockdown. (A) Wound healing assay (a and b), Transwell migration assay (c) and invasion assay (d) for T24 and 5637 treated with *control-siRNA* (*si-control*) and *LAPTM5-target-specific-siRNA* (*si-LAPTM5*). Scale bars for (c and d) are 50 μ m. (B) Statistical analysis of wound healing assay (a), suggesting significantly reduced migration rate (%) after siRNA treatment in T24 and 5637 cells. Statistical analysis of Transwell migration assay (b) and invasion assay (c), suggesting significantly reduced migrated cell number of T24 and 5637 cells after siRNA treatment. All values shown are mean \pm SD of triplicate measurements and repeated three times with similar results. * $p < 0.05$ and ** $p < 0.01$. (C) Western blot analysis of phosphorylated ERK1/2 (p-ERK1/2), total ERK1/2 (t-ERK1/2), phosphorylated p38 (p-p38) and total p38 (t-p38) in the T24 and 5637 cells by siRNA treatment. GAPDH was used as a loading control. (D) Western blot analysis for proteins involved in EMT regulation, revealing that E-cadherin was strongly increased. In contrast, N-cadherin, β -catenin, slug and claudin-1 were considerably decreased after siRNA treatment. GAPDH abundance was used as a loading control.

progression (26), invasion and migration (27). Consistently, our results showed that *LAPTM5* knockdown resulted in an increase of E-cadherin and decrease of N-cadherin, β -catenin and Slug in BCa cells (Fig. 5).

Another important phenomenon observed was that reduced *LAPTM5* exhibited a negative effect on cell proliferation. Since cell proliferation was influenced by cell cycle and apop-

tosis, we found that BCa cells lacking *LAPTM5* significantly induced G0/G1 cell cycle arrest, but apoptotic rate of BCa cells showed no significant alteration. Proteins involved in cell cycle regulation, such as cyclin A1/2, cyclin D1 and CDK2/4, were inhibited by *LAPTM5* knockdown.

Our microarray analysis also suggested that MAPK signaling pathway was linked with bladder cancer through

regulating the cell cycle (3). Recent study reported that the LAPTM5 protein is a positive regulator of MAPK signaling pathway in macrophages (19). Similarly, our study also showed that p-ERK1/2 and p-p38 play important roles in regulating cell proliferation, survival and apoptosis (28) via connecting extracellular stimuli from cell membrane to nucleus were substantially downregulated after *LAPTM5* knockdown. It is known that MAPK family members participate in regulating cell cycle in various manner (29). ERK mainly promotes progression of G0/G1 to S phase and p38 primarily regulates G2 checkpoint (30,31). Meloche and Pouyssegur reported that activation of ERK1/2 could regulate the progression of G1 to S phase by targeting cyclin D1 (29). Our results also revealed that *LAPTM5* knockdown induced cell cycle arrest, which was confirmed by downregulation of related protein (cyclin D1 and CDK2/4) and upregulation of their upstream proteins p-GSK-3 β /t-GSK3 β (Fig. 4). The above results suggested that cell cycle arrest induced by *LAPTM5* knockdown may have a connection with MAPK signaling pathway in bladder cancer.

In conclusion, our results are the first to reveal that down-regulation of *LAPTM5* inhibited migration and invasion by suppressing EMT markers and reduced proliferation in BCa cells. Moreover, this process may be partially connected with the alteration of MAPK signal pathway.

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