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Original Articles

Polypyrimidine tract binding protein 1 promotes lymphatic metastasis and proliferation of bladder cancer via alternative splicing of MEIS2 and PKM

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ABSTRACT

Lymph node (LN) metastasis is the leading cause of bladder cancer-related mortality. Splicing factors facilitate cancer progression by modulating oncogenic variants, but it is unclear whether and how splicing factors regulate bladder cancer LN metastasis. In this study, Polypyrimidine tract binding protein 1 (PTBP1) expression was found to relate to bladder cancer LN metastasis, and was positively correlated with LN metastasis status, tumor stage, histological grade, and poor patient prognosis. Functional assays demonstrated that PTBP1 promoted bladder cancer cell migration, invasion, and proliferation *in vitro*, as well as LN metastasis and tumor growth *in vivo*. Mechanistic investigations revealed that PTBP1 upregulated MEIS2-L variant to promote metastasis and increased expression of PKM2 variant to enhance proliferation by modulating alternative mRNA splicing. Moreover, overexpression of MEIS2-L or PKM2 could rescue the oncogenic abilities of bladder cancer cells and the expression of MMP9 or CCND1 respectively after PTBP1 knockdown. In conclusion, our data demonstrate that PTBP1 induces bladder cancer LN metastasis and proliferation through an alternative splicing mechanism. PTBP1 may serve as a novel prognostic marker and therapeutic target for LN-metastatic bladder cancer.

1. Introduction

Bladder cancer is one of the most common malignancies in the world, with approximately 429,800 newly diagnosed cases and 165,100 deaths annually [1]. Bladder cancer is a clinically heterogeneous malignancy represented by two subtypes, including non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC); NMIBC usually recurs but rarely progress, while MIBC often progress and is associated with poor long-term survival [2]. Compared to MIBC lacking lymph node (LN) metastasis, the mortality rate of MIBC with LN metastasis rises from 18.6% to 77.6% within 5 years, even when the MIBC is treated with radical cystectomy [3]. LN metastasis is a complex multistep process involving the spread, transportation, settlement, and colonization of tumor cells into and within the lymph nodes [4], and effective methods to diagnose LN metastasis in bladder cancer have been established in our previous study [5,6], but the molecular mechanisms of bladder cancer LN metastasis still remain largely unknown. Therefore, it is essential to explore novel molecular mechanisms underlying bladder cancer LN metastasis to identify new targets and solutions for bladder cancer diagnosis and therapy.

Alternative splicing (AS) is a critical step in the posttranscriptional regulation of gene expression; AS expands the proteomic complexity from a limited gene repertoire and plays a significant role in normal development and in various human diseases [7,8]. RNA–protein

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interactions with splicing factors, RNA–RNA base-pairing interactions, and chromatin-based effects that can change or determine splicing patterns are the three common mechanisms of alternative splicing [9]. The aberrant expression of splicing factors facilitates cancer progression by modulating expression of oncogenic variants [8]. Emerging evidence indicates that alternative splicing events can provide selective drug targets and can serve as biomarkers for cancer diagnosis [10].

PTBP1, a member of the heterogeneous nuclear ribonucleprotein (hnRNP) family that contains RNA Recognition Motif (RRM) domains, is a critical regulator of post-transcriptional gene expression by regulating mRNA splicing, RNA metabolism, stability, localization, and translation [11]. PTBP1 is elevated in several types of cancers, including glioblastoma [12], colorectal cancer [13,14], and renal cancer [15], and is involved in cancer progression by facilitating the alternative splicing of numerous gene variants. However, whether and how PTBP1 regulate the progression of bladder cancer remains largely unknown.

In this study, we identified PTBP1 as a splicing factor associated with LN metastatic bladder cancer. PTBP1 was overexpressed in LN-metastatic tumors and primary tumors tissues, and PTBP1 overexpression predicted poor prognosis. PTBP1 promoted metastasis and proliferation of bladder cancer cells both *in vitro* and *in vivo*. Moreover, PTBP1 regulated alternative splicing of MEIS2 and PKM, contributing to cancer progression. Therefore, our findings illustrated the significant role of PTBP1 in the malignant potential of bladder cancer, suggesting that PTBP1 could serve as prognostic biomarker and a promising therapeutic target.

2. Materials and methods

2.1. Human tissue samples

A total of 104 formalin-fixed, paraffin-embedded primary bladder cancer specimens, 25 LN metastatic cancer tissue samples, and 30 normal adjacent tissue (NAT) samples, termed Cohort 1, were obtained with written informed consent from patients who underwent surgery at Sun Yat-sen Memorial Hospital of Sun Yat-sen University (Guangzhou, P.R. China) between July 2007 and January 2017. Tissue microarrays containing 60 bladder cancer specimens, termed Cohort 2, were purchased from US Biomax (catalogue numbers BC12011b). All samples were pathologically confirmed as bladder cancer by two independent pathologists. Ethical consent was approved by Sun Yat-sen University's Committees for Ethical Review of Research involving Human Subjects. Patient demographics and clinical characteristics are provided in Table 1 and S1.

Table 1
Correlation between PTBP1 expression and clinicopathological characteristics of bladder cancer patients from Sun Yat-sen Memorial Hospital.

Characteristic	Patient frequency	PTBP1		Pearson Chi-square	*p* value
		Low	High		
Total	104	52	52		
Gender					
Male	92 (88.5%)	45	47	0.377	0.593
Female	12 (11.5%)	7	5		
Age					
≤65	54 (51.9%)	27	27	0.000	1
> 65	50 (48.1%)	25	25		
Tumor stage					
Ta,T1	41 (39.4%)	29	12	11.636	**0.001**
T2-4	63 (60.6%)	23	40		
Tumor grade					
Low	17 (16.3%)	14	3	7.032	**0.008**
High	87 (83.7%)	38	49		
L.N. status					
Negative	64 (61.5%)	38	26	5.850	**0.016**
Positive	40 (38.5%)	14	26		

2.2. Immunohistochemistry (IHC) staining and scoring analyses

IHC was performed according to a previously described method [16]. Briefly, anti-PTBP1 antibody (ab133734, 1:1000) was used to detect PTBP1 expression in bladder cancer tissue and normal tissue. Anti-PTBP1 and anti-Ki67 antibodies (ZM-0167, 1:500) were used to detect the expression of PTBP1 and Ki67 in murine tumor tissue. Human renal cell carcinoma tissues were used as positive controls for the PTBP1 antibody for IHC staining [15], and negative control samples included replacement of the primary antibody with non-specific IgG (DAKO), as shown in Fig. S1a. The expression of PTBP1 in bladder specimens was blindly quantified by two pathologists according to a staining scoring system. Briefly, as shown in Fig. S1b, the intensity of immunostaining in each sample was graded as negative = 0, weak = 1, moderate = 2 or strong = 3. The proportion of cells staining positively was also assessed as percentage. The staining score was then calculated as the numbers representing intensity times the percentage of cells stained (Score = Intensity × % of positive cells). The samples were classed as low (score < 180) or high (score ≥180) PTBP1 expression. Images were visualized using a Nikon ECLIPSE Ti (Japan) microscope system and processed with Nikon software.

2.3. The GEO and TCGA data mining

Patients' clinical profiles in the CNU bladder cancer cohort are available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13507 [17]. Patients' clinical profiles in the TCGA cohort are available at https://cancergenome.nih.gov/ [18]. Kaplan–Meier survival analysis of PTBP1 in 402-cases in a TCGA cohort was obtained from GEPIA [19] (http://gepia.cancer-pku.cn/index.html). For gene set enrichment analysis (GSEA), normalized expression data were analyzed and visualized with the GSEA software (version 2.2.0, http://www.broadinstitute.org/gsea). The enrichment score (ES) and normalized enrichment score (NES) were calculated for comparison.

2.4. Cell culture

The cell lines used in this study included the human bladder cancer cells UM-UC-3 and T24 (ATCC, Manassas, VA). UM-UC-3 cells were cultured in DMEM (Gibco, Shanghai, China), and T24 cells were cultured in RPMI 1640 (Gibco, Shanghai, China). All media was supplemented with 10% FBS (Biological Industries, 04-001-1ACS) and 1% penicillin/streptomycin. Cells were grown in a humidified atmosphere of 5% CO_2 at 37 °C. All cell lines used in this study tested negative for mycoplasma contamination.

2.5. RNA interference

SiRNA oligonucleotides targeting PTBP1, PKM2, MEIS2-L and negative control siRNA were purchased from GenePharma (Shanghai, China), and listed in Table S6. SiRNA transfections were performed with 75 nM siRNA and Lipofectamine RNAimax (Life Technologies), as previously described [20].

2.6. Lentivirus transduction

To establish stable overexpression and knockdown cell lines, full length PTBP1 or shRNA sequences that specifically target PTBP1 were cloned into the pCDH-CMV-MCS-EF1-Puro or pLKO.1-Puro vectors, respectively. Bidirectional sequencing was performed to verify the correct sequences. The sequences of all shRNAs are listed in Table S6. Lentivirus production and infection were conducted as previously described [20].

2.7. In vitro cell wound healing, migration, and invasion assays

Wound healing assays and Transwell assays were performed to detect cell migration and invasion. The details were described in our previous study [15].

2.8. Cell proliferation assays

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the colony formation assay were performed to detect cell viability. For the colony formation assay, 10 days after seeding cells in a 6-well plate, the clones were washed with PBS and stained with crystal violet for approximately 20 min. Finally, the clones were imaged and quantified. Cell cycle analysis and ethynyl deoxyuridine (EdU) assays were used to detect cell populations at different phase. The details were described in our previous study [21].

2.9. RNA isolation and quantitative RT-PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen), according to manufacturer's instruction, and was used as template for reverse transcription with the PrimerScript RT-PCR kit (TaKaRa Biotechnology, Dalian, China). Quantitative RT-PCR was conducted using a standard SYBR Green PCR kit (Roche) and protocol with a LightCycler 96 real-time instrument (Roche). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The expression of the GAPDH gene was used as an internal control. All specific primers used for quantitative RT-PCR are listed in Table S7.

2.10. Western blotting

Western blot analysis was performed as previously described [21]. Primary antibodies specific to PTBP1 (ab133734, 1:1000), PKM1 (15821-1-AP, 1:500), PKM2 (ab150377, 1:1000), MMP9 (ab38898, 1:1000), cyclin D1 (92G2, 1:1000), and GAPDH (ab8245, 1:1000) were used. After probing with primary antibodies, membranes were incubated with the appropriate goat anti-rabbit or anti-mouse secondary antibody (Cell Signaling Technology). Protein bands were visualized using enhanced chemiluminescence.

2.11. In vivo popliteal LN metastasis and tumorigenesis assay

All procedures involving animals were approved by the Institute Animal Care and Use Committee of Sun Yat-sen University, and the approval number is 170429. Male BALB/c nude mice (4–5 weeks old) were purchased from the Experimental Animal Center of Sun Yat-sen University and housed in SPF barrier facilities. Each group included 10 mice. Lentivirus-transduced UM-UC-3 cells (3×10^6 cells) stably expressing firefly luciferase were inoculated into the foot-pads of experimental mice. Lymphatic metastasis was monitored and imaged with a bioluminescence imaging system (PerkinElmer, IVIS Spectrum Imaging System) four weeks after tumor cell injection. Primary tumors and popliteal LNs were enucleated and embedded in paraffin. The LN volumes were calculated using the following formula: LN volume (mm3) = (length [mm]) × (width [mm])^2 × 0.52. The FFPE samples were analyzed by IHC with anti-luciferase antibodies (ab21176, 1:2000). Images were captured using a Nikon ECLIPSE Ti (Japan) microscope system and processed with Nikon software.

The tumorigenesis assay was performed as previously described [16]. A total of 3×10^6 cells were injected subcutaneously on the right or left side of the dorsum (n = 5). Mice were euthanized four weeks post-implantation, and tumors were surgically dissected. The tumor specimens were fixed in 4% paraformaldehyde.

2.12. RNA sequencing analysis

Cells were transfected with PTBP1 (mixture of siRNA-1 and -2) or control siRNA for 48 h (n = 2). Then, total RNA was extracted from cells using TRIzol (Invitrogen). Library construction and sequencing were performed by Annoroad Gene Technology (Beijing, China). The libraries were sequenced on an Illumina HiSeq 2500 platform and 100-bp paired-end reads were generated. *ASprofile* was used to analyze the differentially alternative splicing events. All primary data in RNA sequencing (RNA-seq) analysis have been uploaded to the Gene Expression Omnibus (GSE79832).

2.13. RNA immunoprecipitation

RNA immunoprecipitation (RIP) was performed using the EZ-Magna RIP kit (Millipore), according to the manufacturer's instructions and described previously [20]. In brief, 1×10^7 cells were lysed with RIP lysis buffer with one freeze-thaw cycle. Cell extracts were co-immunoprecipitated with anti-PTBP1 (ab133734), and the retrieved RNA was subjected to real-time qPCR analysis. Normal rabbit IgG was used as a negative control. For real-time qPCR analysis, U6 was used as a non-specific control.

2.14. RNA pull down

The RNA pull down assay was performed using a Magnetic RNA-Protein Pull-down Kit (Thermo Scientific), according to the manufacturer's instructions and described previously [22]. Samples were separated using electrophoresis, and PTBP1-specific bands were identified by western blot. Beads alone were used as a negative control, and GAPDH was used as a non-specific control. The oligos for RNA pull down are listed in Table S8.

2.15. Statistical analyses

Data are presented as the mean ± SD from three independent experiments. Two-tailed Student's t-tests and one-way analysis of variance (ANOVA), followed by Dunnett's tests for multiple comparisons, were used to evaluate the data. Pearson's chi-square test was used to analyze clinical variables. Spearman's correlation analysis was performed to determine the correlation between two variables. Cumulative survival time was calculated using the Kaplan–Meier method and analyzed by the log-rank test. A multivariate Cox proportional hazards model was used to estimate the adjusted hazard ratios and 95% confidence intervals (CIs), and to identify independent prognostic factors. All statistical analyses were performed with SPSS 19.0. A difference was considered to be statistically significant at $^*p < 0.05$ and $^{**}p < 0.01$.

3. Results

3.1. PTBP1 overexpression correlates with bladder cancer LN metastasis and predicts disease prognosis outcome

To identify important splicing factors in bladder cancer progression, 14 previously reported cancer-critical splicing factor genes in Cancer Gene Census were selected [23,24], and their expression was examined in LN-metastatic and primary bladder tumors and matched adjacent normal tissues from Cohort 1 by quantitative reverse-transcription PCR (qRT-PCR) analysis (Fig. 1a). Intriguingly, PTBP1 was the most significant gene, which was overexpressed not only in primary tumor tissue compared to matched adjacent normal tissue, but also in LN-metastatic tumors compared to primary tumors (Fig. 1b and S1c). PTBP1 protein expression level was further investigated in 104 cases from Cohort 1 by IHC (Fig. 1c). Consistent with the RNA expression data, PTBP1 protein expression level was low in normal adjacent tissue, but increased as disease severity progressed in LN-negative tumor

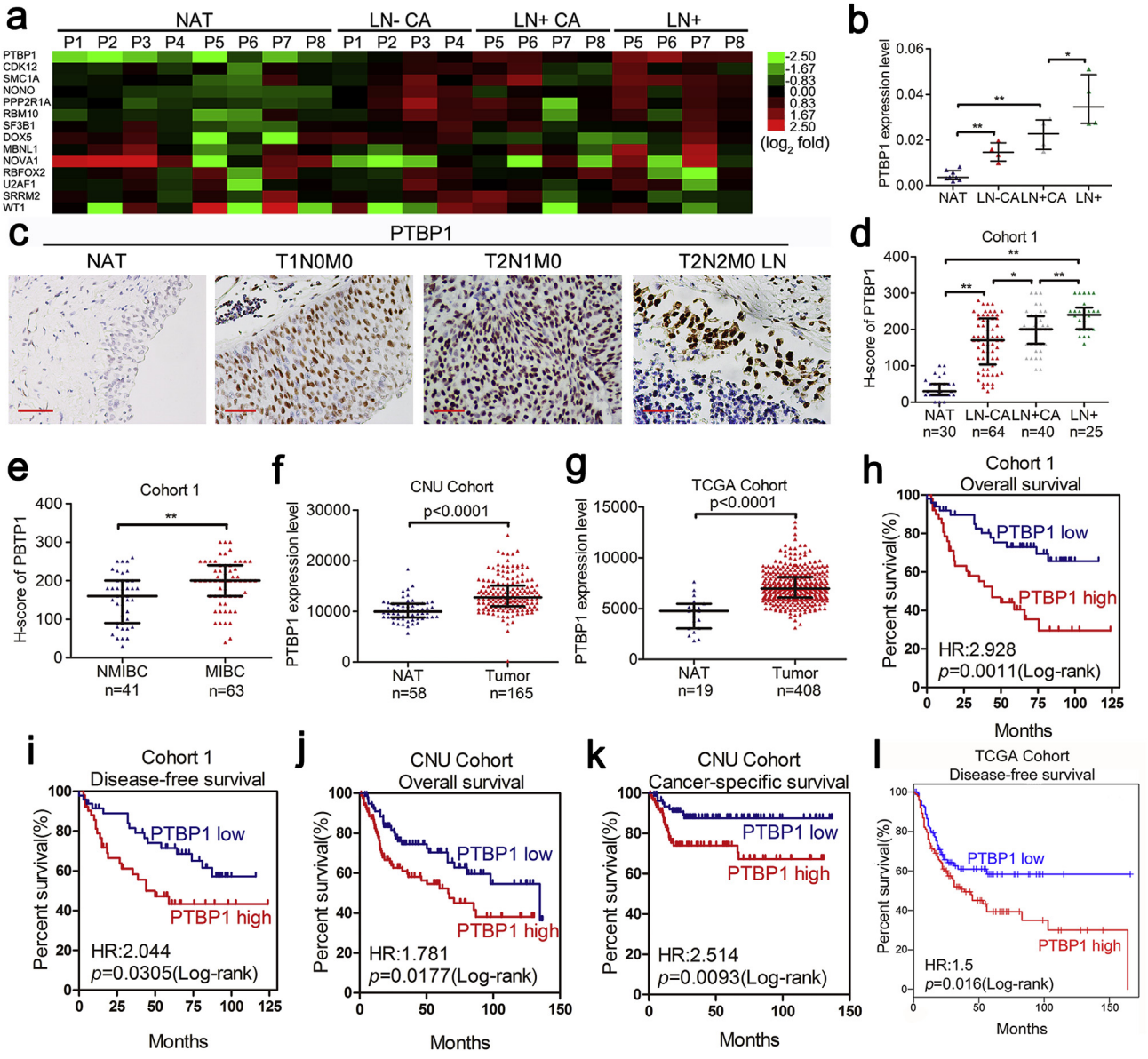

Fig. 1. PTBP1 expression correlates with bladder cancer LN metastasis and predicts poor prognosis. a. Unsupervised hierarchical clustering of splicing factors expressed in LN metastatic, LN-positive, LN-negative cancer, and normal adjacent tissues detected by qRT-PCR. The color bar represents the intensity scale, generated by a log2 transformation. **b.** qRT-PCR analysis of PTBP1 expression in the evaluated tissues. **c.** Representative IHC images of PTBP1 expression in paraffin-embedded NAT and tumor sections of bladder cancer with or without LN metastasis (n = 104). Scale bars: red, 50 μm. **d.** IHC staining of Cohort 1 showed the expression levels of PTBP1 across NAT, LN negative, LN positive, and LN metastatic tumors tissues. **e.** PTBP1 expression was detected in NMIBC and compared with MIBC in Cohort 1. **f, g.** PTBP1 expression was detected in NAT and compared with bladder cancer tissues in the CNU and TCGA Cohorts. **h, i.** Kaplan-Meier curves for OS and DFS of bladder cancer patients with high vs. low expression of PTBP1 in Cohort 1. The patients were divided into PTBP1-low (n = 52) and PTBP1-high groups (n = 52). **j, k.** Kaplan-Meier curves for OS and cancer-specific survival (CSS) of bladder cancer patients with high vs. low expression of PTBP1 in the CNU Cohort. The patients were divided into PTBP1-low (n = 78) and PTBP1-high groups (n = 87). **l.** Kaplan-Meier curves for DFS of bladder cancer patients with high vs. low expression of PTBP1 in the TCGA Cohort. The patients were divided into PTBP1-low (n = 165) and PTBP1-high groups (n = 237). Statistical significance was assessed using two-tailed t-tests or one-way analyses of variance (ANOVA). Scale bars: 50 μm. The error bars represent standard deviations of three independent experiments. *p < 0.05 and **p < 0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

tissue, LN-positive tumor tissue, and LN-metastatic tumor tissue (Fig. 1d). Additionally, PTBP1 was overexpressed in MIBC compared with NMIBC (Fig. 1e), as well as in high-grade bladder cancer compared with lower-grade tumors (Fig. S1d). Analysis of a 165-case CNU (Chungbuk National University) Cohort (GSE13507) and a 408-case cohort from The Cancer Genome Atlas (TCGA) database also showed that PTBP1 expression was significantly overexpressed in tumor tissues compared with normal adjacent tissues (Fig. 1f and g). Moreover, correlation analysis with clinicopathological features revealed that PTBP1 expression was positively correlated with poor differentiation state, advanced tumor stage, and LN metastasis status (Table 1). This observation was further confirmed in an additional cohort, Cohort 2, consisting of a tissue microarray containing 60 bladder cancer tissues (Table S1). Kaplan–Meier survival analysis revealed that patients with high PTBP1-expressing bladder cancers had significantly shorter overall survival (OS) and disease-free survival (DFS) in Cohort 1 ($p = 0.0011$ and $p = 0.0305$, Fig. 1h and i). The association of high PTBP1 expression with poor clinical outcome was confirmed by analysis of the CNU Cohort (Fig. 1j and k) and the TCGA Cohort (Fig. 1l). Furthermore, univariate analysis indicated that PTBP1 expression was significantly

Table 2
Univariate and multivariate analysis of factors associated with overall survival of bladder cancer from Sun Yat-sen Memorial Hospital.

Variable	Univariate			Multivariate		
	HR	95% CI	*p*	HR	95% CI	*p*
Age, years (> 65/≤65)	1.480	0.799–2.739	0.212			NA
Gender (Female/Male)	0.905	0.355–2.308	0.834			NA
Histological grade (High/Low)	14.710	2.015–107.39	**0.008**	6.226	0.796–48.693	0.081
Tumor stage (T2–T4/Ta–T1)	6.126	2.564–14.637	**< 0.001**	3.198	1.287–7.946	**0.012**
Nodal metastasis (N1−N2/N0)	3.787	2.011–7.132	**< 0.001**	1.701	0.812–3.564	0.159
PTBP1(High/Low)	3.030	1.573–5.835	**0.001**	1.960	1.009–3.818	**0.048**

Univariate and multivariate analysis. Cox proportional hazards regression model. Variables associated with survival by univariate analyses were adopted as covariates in multivariate analyses. Significant P-values are shown in bold font. HR > 1, risk for death increased; HR < 1, risk for death reduced.

associated with OS and DFS in Cohort 1 (Table 2 and S2); this association was corroborated in the CNU Cohort (Tables S3 and 4). Multivariate Cox regression analysis demonstrated that high PTBP1 expression in bladder cancer tissue was an independent prognostic factor for OS in Cohort 1 (Table 2). Collectively, these data demonstrate that PTBP1 is a splicing factor that is related to bladder cancer LN-metastasis, as PTBP1 expression is associated with LN metastasis status, tumor stage, and histological grade, indicating that PTBP1 may serve as a marker of poor prognosis in bladder cancer.

3.2. PTBP1 promotes metastatic behavior of bladder cancer cells in vitro and LN metastasis in vivo

To investigate the role of PTBP1 in bladder cancer metastasis, T24 and UM-UC-3 bladder cancer cell lines were transfected with siRNAs targeting PTBP1 or were established to stably overexpress PTBP1 by lentivirus. The efficiency of PTBP1 knockdown and overexpression was confirmed by qRT-PCR and western blotting (Fig. 2a and b). Wound healing assays showed that downregulation of PTBP1 decreased, whereas upregulation of PTBP1 increased, the migratory speed of T24 and UM-UC-3 cells (Fig. 2c and d). Moreover, PTBP1 knockdown inhibited migration and invasion of bladder cancer cells, whereas the opposite outcome was observed after PTBP1 overexpression (Fig. 2e and f). Collectively, these results indicated that PTBP1 may play an important role in the metastatic behavior of bladder cancer in *vitro*.

To further determine the role of PTBP1 in lymph node metastasis, a popliteal lymph node metastasis model was established *in vivo*. UM-UC-3 bladder cancer cells stably overexpressing PTBP1, or expressing a short hairpin RNA (shRNA) targeting PTBP1, were generated by lentiviral transfection; these cells also stably expressed firefly luciferase, and were inoculated into the foot-pads of nude mice (Fig. 3a and b). Interestingly, PTBP1 knockdown significantly inhibited metastasis of bladder cancer cells to the LNs, while PTBP1 overexpression promoted LN metastasis, as was determined by *in vivo* imaging (Fig. 3c and d). Additionally, the mice bearing PTBP1-overexpressing tumors had shorter survival times, while mice bearing PTBP1-knockdown tumors had longer survival times than those in the corresponding control groups (Fig. 3e). The volume of tumors formed by PTBP1-overexpressed cells was larger compared to the corresponding control cells (Fig. 3f and g). In contrast, the tumors formed by the PTBP1-silenced cells were smaller than vector-control cells (Fig. 3f and g). Meanwhile, hematoxylin-eosin (HE) staining and immunostaining of luciferase further validated that PTBP1 facilitated the lymphatic metastasis of bladder cancer cells (Fig. 3h and S2a), as determined by comparing the number of LNs bearing metastases (Table S5). Moreover, the tumors derived from the PTBP1-silencing group exhibited decreased expression of metastasis marker MMP9, but MMP9 was upregulated in tumors from the PTBP1-overexpressing group (Figs. S2b and c). Pearson correlation analysis revealed that PTBP1 expression is positively correlated with MMP9 expression (Fig. S2d). Taken together, these results indicate that PTBP1 plays a key role in lymph node metastasis of bladder cancer.

3.3. PTBP1 enhances proliferation of bladder cancer cells in vitro and tumorigenesis in vivo

To investigate the function of PTBP1 in the proliferation of bladder cancer cells, we first conducted MTT and colony formation assays. PTBP1 knockdown inhibited the cell viability and colony formation of T24 and UM-UC-3 cells, whereas the PTBP1 overexpression increased viability and colony formation (Fig. 4a–d). Next, we performed flow cytometry and EdU assays to determine whether PTBP1 is involved in cell cycle regulation. Silencing of PTBP1 increased the proportion of cells in the G0/G1 phase and reduced the proportion of cells in the S phase, while PTBP1 overexpression had the opposite effect (Fig. 4e and f, S3a and b). Consistent with these data, the EdU assays revealed that PTBP1 significantly regulated the cell population in S phase (Fig. 4g and h, S3c and d). Taken together, these results indicated that PTBP1 accelerates the proliferation of bladder cancer cells by inducing cell cycle progression.

To further evaluate the effects of PTBP1 on bladder cancer cell tumorigenesis *in vivo*, stable PTBP1 knockdown or overexpression UM-UC-3 cells were implanted subcutaneously into nude mice and tumor growth was measured. PTBP1 knockdown inhibited tumor growth, and PTBP1 overexpression enhanced tumor growth, compared to the corresponding control group (Fig. 5a and b). The size and weight of the tumors from the PTBP1 knockdown group were lower than the control group, while PTBP1 overexpression resulted in larger tumors (Fig. 5c and d). Meanwhile, the tumors derived from the PTBP1 knockdown group exhibited lower expression of proliferation marker Ki67, but Ki67 was increased in tumors from the PTBP1 overexpression group (Fig. 5f and g). The expression levels of PTBP1 and Ki67 were further analyzed by Pearson correlation, revealing that PTBP1 expression was positively correlated with Ki67 expression (Fig. 5e). Collectively, these results suggest that PTBP1 promotes the *in vivo* tumorigenesis of bladder cancer cells.

3.4. PTBP1 regulates alternative splicing of MEIS2 and PKM

To further gain insight into the molecular mechanisms underlying the oncogenic role of PTBP1, we performed transcriptomic sequencing of UM-UC-3 cells treated with PTBP1 siRNA or control siRNA. Considering that PTBP1 is a critical splicing factor, we concentrated on AS changes induced by PTBP1 knockdown. We analyzed AS events regulated by PTBP1 knockdown, and identified 566 AS events with significant change ($p < 0.05$) (Fig. 6a). We selected the most significant AS events and validated by these AS events by qPCR using specific primers designed to skip exons (Fig. 6b). We found several significantly changed genes, including myeloid ecotropic insertion site 2 (MEIS2) and pyruvate kinase isozymes (PKM), which are crucial oncogenes in several cancer types [25,26]. Consistent with the transcriptomic sequencing results, PTBP1 overexpression promoted the inclusion of MEIS2 exon 2, while PTBP1 silencing repressed the inclusion of MEIS2 exon 2, displayed as a MEIS2-L/MEIS2-S ratio; the same

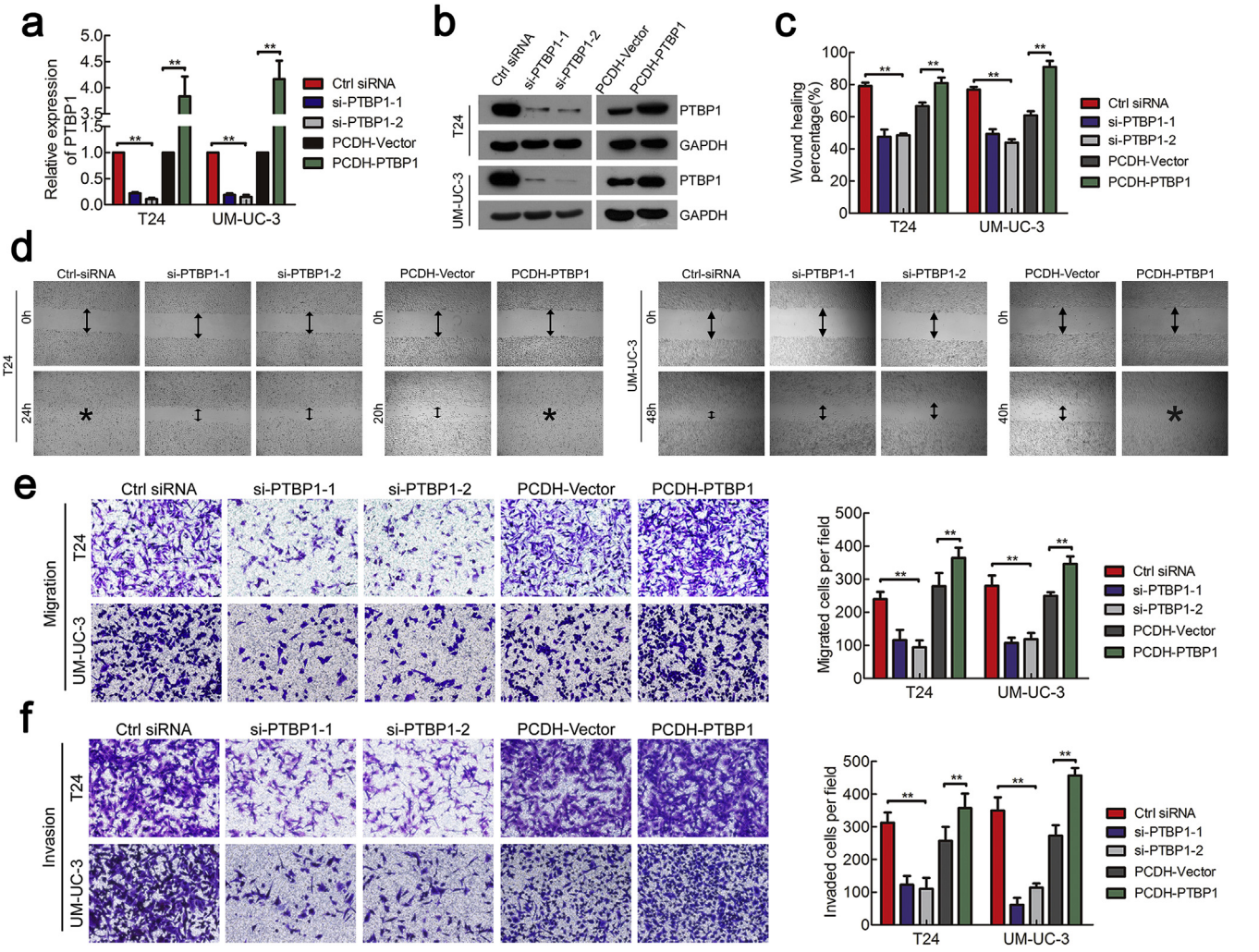

Fig. 2. PTBP1 promotes metastasis of bladder cancer cells *in vitro*. **a, b.** qRT-PCR and western blot analysis of PTBP1 mRNA and protein expression levels in PTBP1-silenced cells, PTBP1-transduced cells, and control cells. **c, d.** Representative images of wound healing assays using T24 and UM-UC-3 cells showing cell motility after knockdown or overexpression of PTBP1; a histogram analysis of cell migration distances are shown. **e, f.** Representative images of migration and invasion assays using T24 and UM-UC-3 cells (left panels) showing cell migration and invasion after knockdown or overexpression of PTBP1; a histogram analysis of migrated or invaded cell counts are shown (right panels). Statistical significance was assessed using two-tailed t-tests or ANOVA. *$p < 0.05$ and **$p < 0.01$.

changes were seen with PKM exon 10 inclusion and exon 9 exclusion, displayed as a PKM2/PKM1 ratio (Fig. 6c–e). Previous studies indicated that some intron-binding proteins can induce intronic structures conducive to the inclusion of the neighboring exon, and PTBP1 frequently functions as a splicing factor by binding to the specific elements of intron [27,28]. To determine whether PTBP1 is directly involved in the regulation of MEIS2 and PKM splicing, RIP assays were conducted in T24 and UM-UC-3 cells. The inronic fragments flanking MEIS2 exon 2 and PKM exon 8 were enriched in PTBP1 RIP, compared to the negative control (Fig. 6f and g). Furthermore, RNA pull down assays revealed direct interactions between PTBP1 and RNA oligomers derived from the PKM or MEIS2 intronic regions. PTBP1 was pulled down by oligomer I8 of PKM and 2-2 of MEIS2 containing the PTBP1 consensus binding sequence (UCUUC) (Fig. 6h). Mutation of the PTBP1 binding site from UCUUC to ACAAC abolished binding between the MEIS2 intron and PTBP1 (Fig. 6i), further confirming the specific interaction between MEIS2 and PTBP1. Moreover, Pearson correlation analysis between PTBP1, MEIS2-L/MEIS2-S ratio, and PKM2/PKM1 ratio, as determined by qPCR, revealed that PTBP1 expression was positively correlated with MEIS2-L/MEIS2-S and PKM2/PKM1 ratio (Fig. 6j and k). Taken together, these data indicated that PTBP1 regulates alternative splicing of PKM and MEIS2 by directly binding to specific introns of these mRNA transcripts.

3.5. MEIS2-L and PKM2 isoforms are required for PTBP1-induced metastasis and proliferation

Given the regulation of MEIS2 and PKM splicing by PTBP1, we then investigated whether PTBP1 induces bladder cancer progression in a MEIS2 or PKM dependent manner. Firstly, PKM2 silencing markedly repressed proliferation of bladder cancer cells, while silencing of MEIS2-L did not (Fig. 7a and b, S4a-c). On the other hand, MEIS2-L knockdown significantly inhibited the migration and invasion capacities of bladder cancer cells, while PKM2 knockdown did not (Fig. 7c and d, S4d-g). These results indicate that PKM2 and MEIS2-L likely contribute to different functions of bladder cancer cells. Interestingly, the decrease in proliferation and colony formation of bladder cancer cells seen with PTBP1 knockdown was rescued by PKM2 overexpression (Fig. 7e–g). On the other hand, overexpression of MEIS2-L rescued the decreased migration and invasion of bladder cancer cells induced by PTBP1 knockdown (Fig. 7h–j). Moreover, we analyzed the expression of MEIS2-L by qPCR in our clinical samples. We found that the expression of MEIS2-L was upregulated in bladder cancer tissue compared with NAT (Fig. S4h). Additionally, MEIS2-L expression level was much

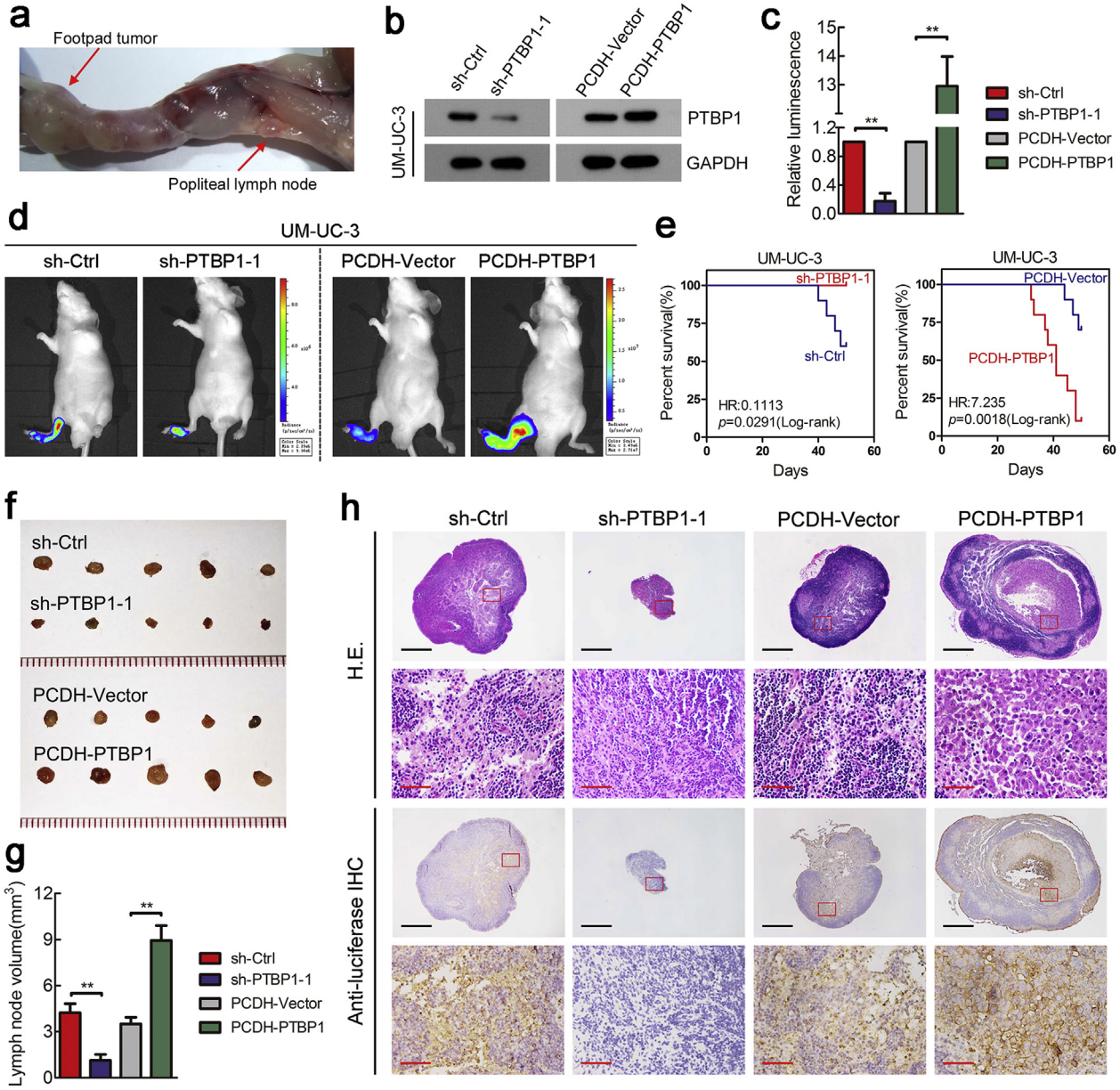

Fig. 3. PTBP1 facilitates LN metastasis of bladder cancer cells *in vivo*. **a.** Representative images of the nude mouse model of popliteal LN metastasis. The indicated UM-UC-3 cells were injected into the footpads of the nude mice, and the popliteal LNs were enucleated and analyzed. **b.** Western blot analysis of PTBP1 expression levels in stably PTBP1-silenced or PTBP1-transduced cells and control cells. **c, d.** Representative images of bioluminescence (popliteal LNs) and histogram analysis of popliteal LN metastasis in the indicated cell groups (n = 10 per group). **e.** Kaplan-Meier survival analysis of the mice (n = 10 per group) that were inoculated with PTBP1 knockdown or overexpressing UM-UC-3 cells compared with the corresponding control cells. **f, g.** Representative images of dissected popliteal LNs and histogram analysis of the LN volume. **h.** Representative images of HE and IHC staining confirming the LN status (n = 10). Scale bars: black, 500 μm; red, 50 μm *p < 0.05 and **p < 0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

higher in LN-positive tumor tissue than LN-negative tissue, as well as in MIBC than NMIBC (Figs. S4i and j). These data indicate that MEIS2-L plays a key role in bladder cancer LN metastasis, which is consistent with the oncogenic functions of MIES2-L in bladder cancer cells.

To further elucidate the molecular mechanisms underlying PTBP1-induced progression in bladder cancer, transcriptomic sequencing of PTBP1-silenced and control UM-UC-3 cells was analyzed. We identified 1353 genes exhibiting significant expression changes (fold change > 2.0, Fig. 8a). We found that several genes that play critical roles in proliferation and lymphatic metastasis were significantly down-regulated in PTBP1-silenced cells, including as MMP9 and CCND1. Intriguingly, Gene Set Enrichment Analysis (GSEA) for genes differentially expressed between low and high PTBP1 expression (from the TCGA dataset) indicated that gene signatures reported to be correlated with metastasis and proliferation were positively enriched in the high PTBP1 expression group ($p < 0.001$, Fig. S5a and b). The expression of MMP9 and CCND1 was upregulated in PTBP1-transduced cells and downregulated in PTBP1-silenced cells, at both the mRNA and the protein levels (Fig. 8b and c). To explore whether PTBP1 regulates MMP9 and CCND1 by splicing of MEIS2-L and PKM2, we overexpressed MEIS2-L or PKM2 in PTBP1 knockdown cells and control cells. We found that PKM2 overexpression abolished downregulation of CCND1 expression induced by PTBP1 knockdown (Fig. 8d and f), and MEIS2-L overexpression rescued MMP9 expression change caused by PTBP1 knockdown (Fig. 8e and g). Collectively, these results demonstrate that PTBP1 promotes lymphatic metastasis and proliferation of bladder cancer by alternative splicing in a MEIS2-L and PKM2 dependent manner (Fig. 9).

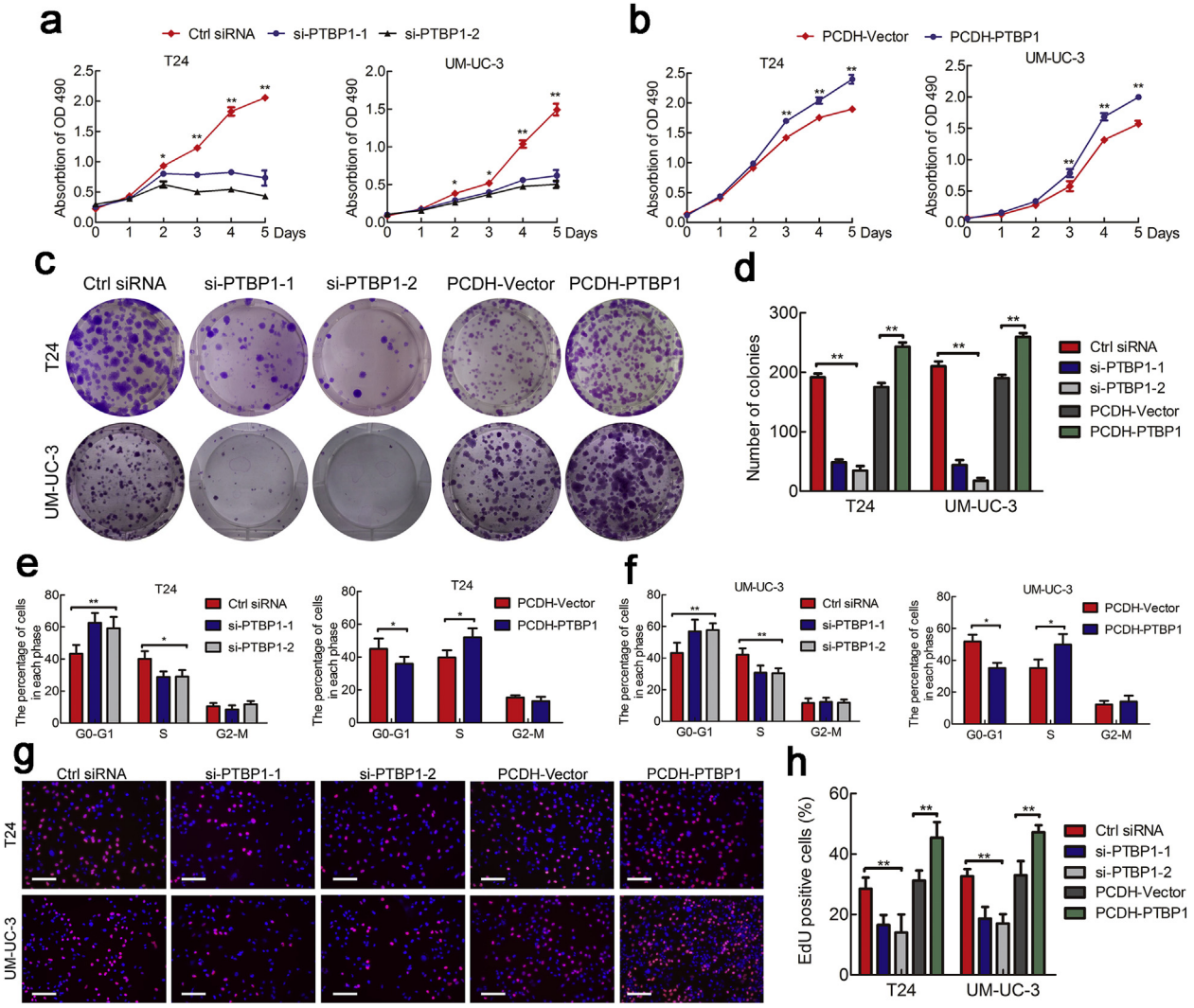

Fig. 4. PTBP1 enhances proliferation of bladder cancer cells *in vitro*. **a, b**. Cell viability was evaluated in PTBP1 knockdown or overexpressing T24 and UM-UC-3 cells. **c, d.** Colony formation assays were performed in PTBP1 knockdown or overexpressing T24 and UM-UC-3 cells. **e, f.** Flow cytometry analysis of T24 and UM-UC-3 cells transfected with PTBP1 siRNA or stably PTBP1-overexpressing, compared with corresponding control cells. The percentages (%) of cell populations at different stages of the cell cycle are listed in the panels. **g, h.** EdU assay measurement of the cell population in the S phase and a histogram analysis of EdU-positive cell counts are shown. Blue, nucleus; red, S-phase cells. Scale bars: white, 100 μm *$p < 0.05$ and **$p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

The prognosis for bladder cancer patients with LN metastasis is poor and options for treatment of metastatic bladder cancer are currently limited [29]. Therefore, elucidation of molecular mechanisms that underlying LN metastasis may facilitate clinical prevention and therapeutic strategies for patients with LN metastatic bladder cancer. Several genes associated with LN metastasis of bladder cancer have been studied, including NF-κB, DANCR and VEGFR-3 [30–32]. To the best of our knowledge, this is the first study to systematically evaluate the role of splicing factors in LN metastasis of bladder cancer. In this study, we report that the splicing factor PTBP1 is overexpressed in LN metastatic bladder cancer. We found that expression of PTBP1 is associated with poor clinical prognosis. Overexpression of PTBP1 enhanced metastasis and proliferation of bladder cancer cells *in vitro* and *in vivo*. Mechanistically, PTBP1 induced alternative splicing of MEIS2 and PKM by directly binding to the specific intron sequences.

Alternative splicing is a key biological process that enables a single gene to code for different proteins that exert different functions. Emergent evidence indicates that aberrant splicing factors can generate oncogenic variants that contribute to the incidence and development of cancer [33]. The hnRNP family of critical splicing factors plays a significant role in normal physiological processes and in cancer development [34]. In a previous study, we reported that hnRNPK is correlated with poor prognosis in bladder cancer and regulates the proliferation and chemoresistance of bladder cancer cells [16]. PTBP1, also known as hnRNPI, has been studied in a number of different cancers, and PTBP1 expression predicts poor prognosis and contributes to tumorigenesis and cancer progression [12,35]. While we were submitting this paper, we noted a recent study reported that PTBP1 expression correlates with non-muscle invasive bladder cancer progression [36]. However, whether and how PTBP1 functions in the LN metastasis of bladder cancer remains unknown. Our data demonstrated a stepwise increase in the expression of PTBP1 from adjacent normal tissues, to LN negative bladder cancer, to LN positive bladder cancer, and finally to bladder cancer lymphatic metastasis. PTBP1 expression level is positively correlated with LN metastasis status, tumor stage and histological grade, and predict poor prognosis in several independent bladder cancer cohorts. Multivariate analysis revealed that PTBP1 expression is an independent predictor of survival outcomes for patients with bladder

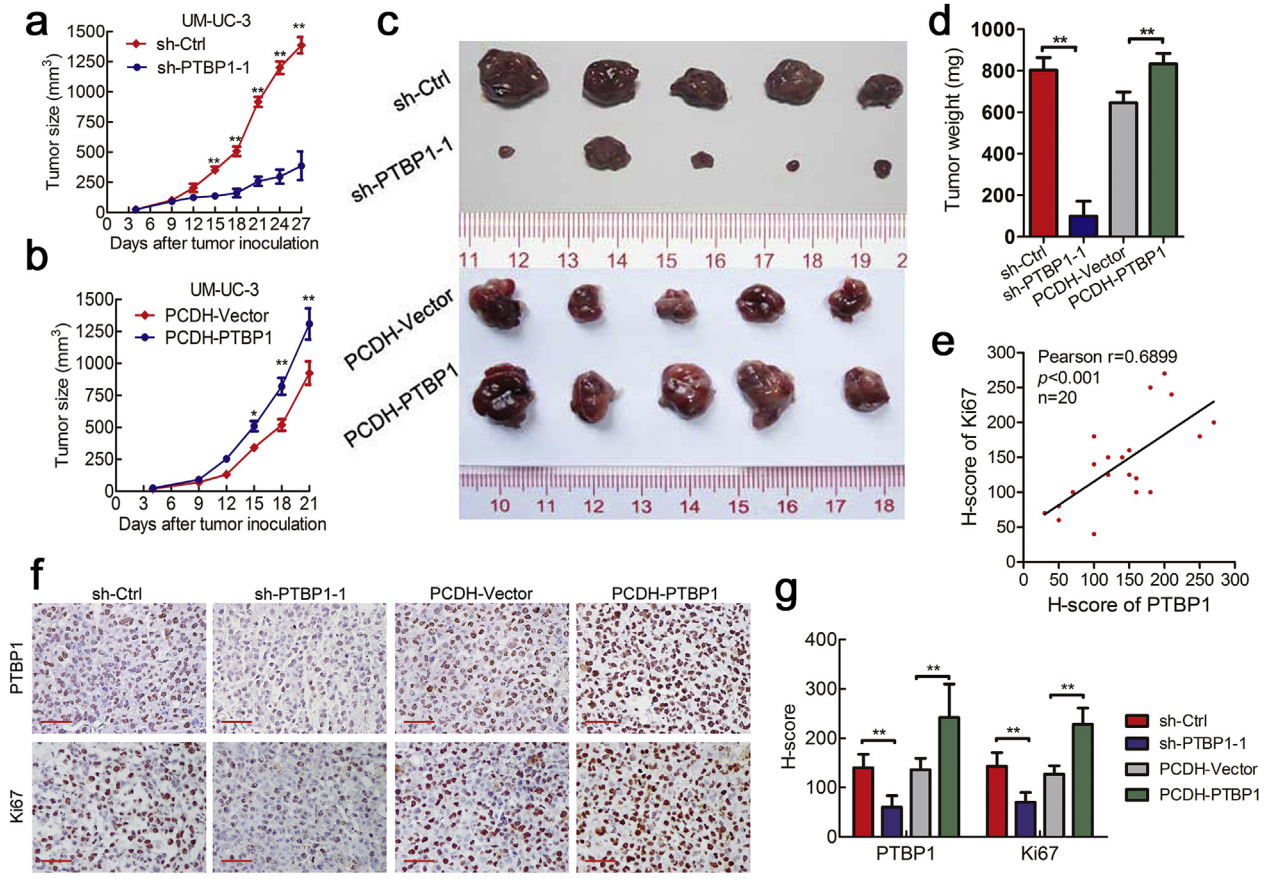

Fig. 5. PTBP1 drives tumorigenesis of bladder cancer cells *in vivo*. **a, b.** Tumor growth curves are summarized in the line chart. The average tumor volume is expressed as the mean ± SD of five mice. **c.** Representative images of the tumors of PTBP1 knockdown or overexpression groups and their respective controls. **d.** Tumor weights were measured after the tumors were surgically dissected. **e, f, g.** IHC examination of tumor PTBP1 and Ki67 expression. Histogram shows the H-score in PTBP1 knockdown or overexpression groups and control group. Pearson correlation analysis was performed between PTBP1 and Ki67 expression. Scale bars: red, 50 μm*p < 0.05 and **p < 0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

cancer. Consistently, PTBP1 overexpression was found to correlate with poor prognosis in other malignancies, including glioblastoma [12], ovarian cancer [37], and clear-cell renal cell carcinoma [15]. Taken together, these data indicate that PTBP1 may serve as a biomarker for LN metastasis and poor prognosis in bladder cancer.

Previous study revealed that brain-specific miR-137 and muscle-specific miR-206, binds to 3' UTR of PTBP1 to repress its expression, inducing the PKM1 expression [38]. And PTBP1 is downregulated by miR-124 during neurogenesis [39]. Moreover, in colorectal cancer, miR-124 decrease PTBP1 expression by directly interacting with its binding site, causing the switching of PKM isoform to affect the tumor proliferation [14]. These studies suggest that PTBP1 is predominately regulated by mircoRNAs both in normal tissues and tumor. On the other hand, PTBP1 is involved in multiple steps of post-transcriptional modulation of gene expression, including alternative splicing [25]. While PTBP1 is expressed in most tissues, PTBP2, also known as brain or neuronal PTB protein, is more tissue specificity [40]. Previous studies show that PTBP2 is expressed with high level in brain and testis, contributing to neuronal differentiation and spermatogenesis [41,42]. Additionally, PTBP1 and PTBP2 have been reported to promote proliferation and migration of glioma cell lines through alternative splicing, indicating their key roles in cancer progression [43]. In cancer, PTBP1 regulates the expression of different isoforms of many genes, including PKM, ANXA7 and BCL-X [12,44,45], which contribute to cancer progression. However, the specific genes that are alternatively spliced by PTBP1 in bladder cancer are largely unknown. Herein, through transcriptional sequencing, we report that PTBP1 regulates alternative splicing of MEIS2 and PKM in bladder cancer. A high ratio of PKM2/PKM1 has been observed in multiple cancers, and was found to promote tumorigenesis [44], tumor growth [46–48], chemoresistance [49]; this was confirmed in our study to occur in bladder cancer. Importantly, we discovered that PTBP1 promotes MEIS2 exon 2 inclusion by directly binding to a downstream intron sequence, as verified by RIP and RNA pull down assays. PTBP1 usually functions as a splicing repressor by binding repressively to the splicing silencer elements [27]. Meanwhile, PTBP1 can also activate exon inclusion by binding to the polypyrimidine tract in some cases [12,50]; this was also demonstrated in our study. After binding to its target site, PTBP1 was reported to inhibit recruitment of U2AF65 or regulate base pairing of U2 snRNP, or disrupt interaction of U1 snRNP with spliceosome elements to exert its regulatory function [51–53]; nevertheless, the detailed mechanism for PTBP1 activity in bladder cancer requires further investigation. Moreover, functional assays revealed that MEIS2-L and PKM2 overexpression rescued the decrease in metastasis and proliferation that were induced by PTBP1 knockdown. Overall, our data demonstrate that PTBP1 promotes LN metastasis and proliferation of bladder cancer cells by upregulating MEIS2-L and PKM2 oncogenic variants, suggesting that PTBP1 may serve as a therapeutic target for clinical intervention in LN-metastatic bladder cancer.

MEIS2 is a member of three amino-acid loop extension (TALE) of homeodomain-containing transcription factors that regulates critical functions in cell fate determination during development [54,55]. The C-terminal transcriptional activation domain in MEIS2 is necessary for activation of transcription by homeodomain protein complexes [56]. MEIS2 was reported to be overexpressed in several cancers and serves as an oncogene in neuroblastoma and leukemia [26,57,58]. Moreover,

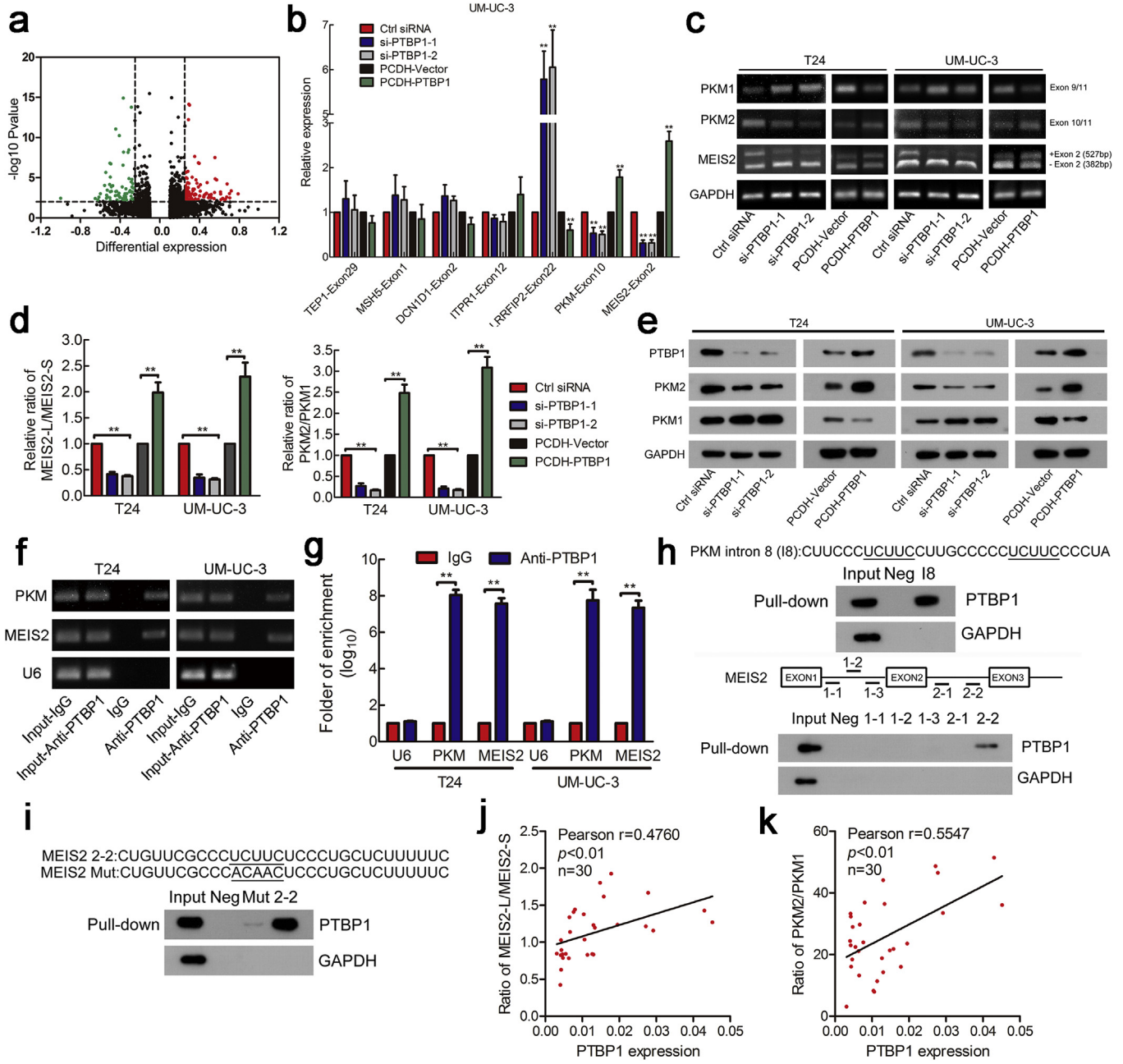

Fig. 6. PTBP1 regulates alternative splicing of MEIS2 and PKM. **a.** Volcano plots displaying the different AS events regulated by PTBP1. Differential expression values are plotted against p-value; red dots and green dots represents the differential exon skipping genes. **b.** Validation of candidate genes by qRT-PCR in UM-UC-3 cells. **c, d.** Analysis of the expression of PKM2, PKM1, MEIS2 and MEIS2-L/MEIS2-S, PKM2/PKM1 ratio in PTBP1 knockdown or overexpressing T24 and UM-UC-3 cells by RT-PCR. **e.** Analysis of the expression of PKM2 and PKM1 in PTBP1 knockdown or overexpressing T24 and UM-UC-3 cells by western blot. **f, g.** qRT-PCR analysis of PKM and MEIS2 from RNA immunoprecipitation assay of T24 and UM-UC-3 cells using anti-PTBP1. RNA enrichment was determined relative to the non-targeting IgG control. U6 was used as a non-specific control. **h.** RNA pull down assay was performed using potential binding sequences in the intron segment of PKM and MEIS2-L pre-mRNA. PTBP1 was detected in protein isolated from UM-UC-3 cells by western blot. GAPDH protein was detected as a non-specific control; beads alone were used as a negative control. **i.** RNA pull down assays were performed using mutation of the potential binding sequence in the intron segment 2-2 of MEIS2-L pre-mRNA. **j, k.** Pearson correlations between expression of PTBP1, MEIS2-L, and PKM2, as assessed by qPCR analysis, from 30 bladder cancer patients. *p < 0.05 and **p < 0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

diverse variants of MIES2 have been implicated in neuroblastoma, and were found to exert different functions and to be associated with tumor stage [26]. In this study, we identified a novel isoform of MEIS2 that facilitates migration and invasion of bladder cancer cells and regulates the expression of MMP9. Interestingly, MMP9 plays a critical role in lymphatic metastasis [59,60], indicating that the important functional axis of PTBP1/MEIS2-L/MMP9 may be dependent on alternative splicing. Therefore, MEIS2 may act as an essential LN metastasis-associated transcriptional factor, and further investigation into the function and mechanism of MEIS2 is merited. In addition, PKM2 upregulation and PKM1 downregulation play important roles in metabolism and growth by enhancing the Warburg effect in cancer [44]. The critical switch of PKM isoforms is regulated by PTBP1 and promotes cancer progression in multiple malignancies [25,61]. Previous study found that PTBP1 regulates PKM mRNA splicing by co-operating with hnRNPA1 and SRSF3 in colon cancer cells [62], which would be further investigated

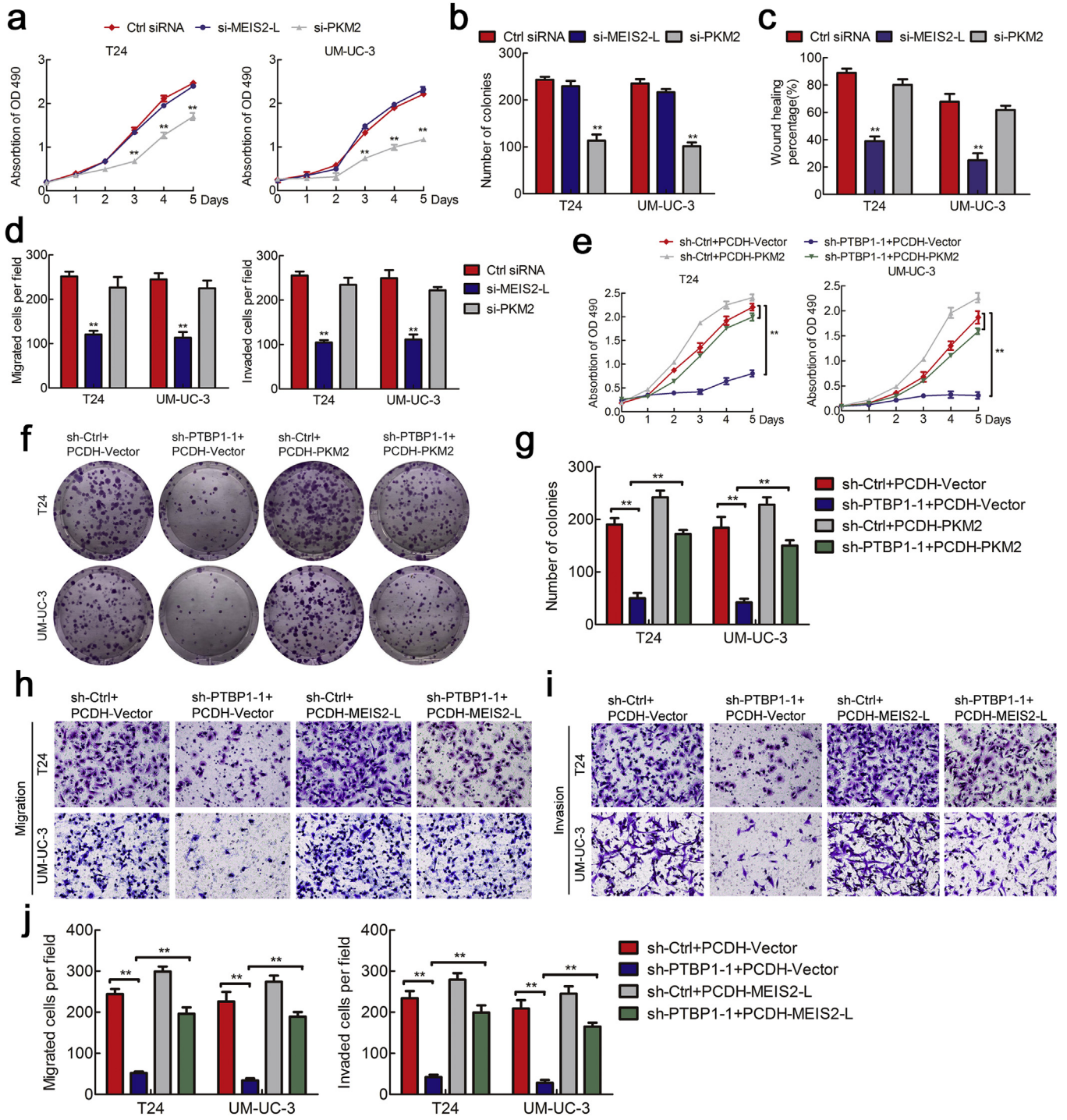

Fig. 7. MEIS2-L and PKM2 isoforms are required for PTBP1-induced metastasis and proliferation. a, b. Cell viability and colony formation were evaluated in MEIS2-L or PKM2 knockdown T24 and UM-UC-3 cells. **c, d.** Cell motility and invasion were evaluated in MEIS2-L or PKM2 knockdown T24 and UM-UC-3 cells. **e, f, g.** Cell viability and colony formation were analyzed using PTBP1 knockdown or control cells combined with PKM2 overexpression. **h, i, j.** Cell migration and invasion were analyzed using PTBP1 knockdown or control cells combined with MEIS2-L overexpression. *p < 0.05 and **p < 0.01.

in bladder cancer in our future study. Meanwhile, in addition to its well-established role in aerobic glycolysis, PKM2 also directly modulates gene transcription and influences protein kinase activity [63]. In the present study, our results indicated that PTBP1-mediated upregulation of PKM2 promotes proliferation of bladder cancer by inducing cell cycle progression from G1 phase to S phase through increasing CCND1 expression. A previous study reported that PKM2 directly binds to and phosphorylates histone H3, which dissociates HDAC3 (histone deacetylase 3) from CCND1 promoter regions, leading to subsequent acetylation of histone H3 and to activation of gene transcription [64]. Therefore, we suggest that PKM2 is upregulated by PTBP1, leading to the induction of CCND1 expression that ultimately causes cell cycle progression [65]. Previous studies have demonstrated that PBTP1 plays an important role in colorectal cancer cell proliferation by regulating cell cycle [66], which is consistent with our study. Collectively, these results demonstrate that both MEIS2-L and PKM2 are regulated by PTBP1 and play critical roles in bladder cancer progression; MEIS2-L and PKM2 may represent novel therapeutic targets in bladder cancer.

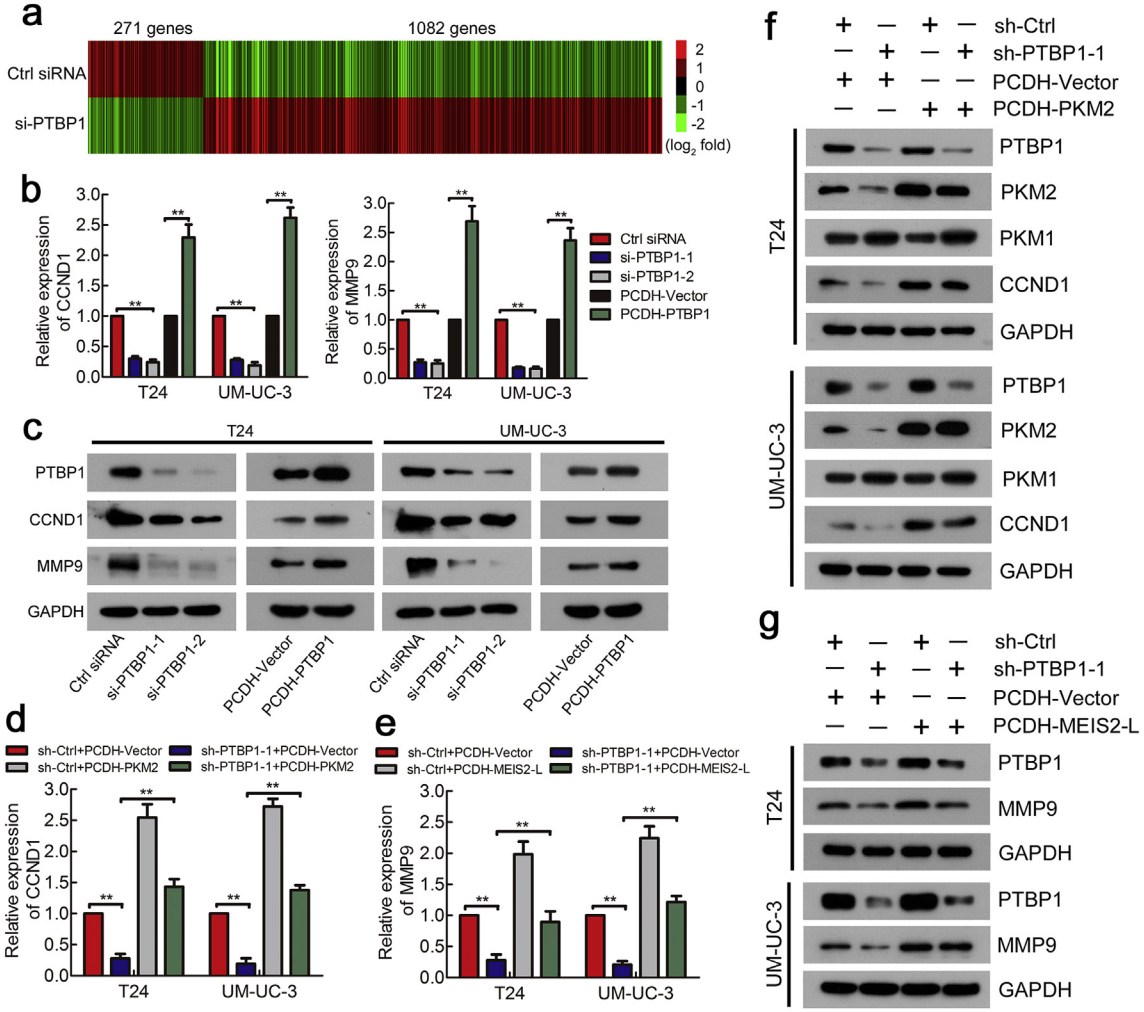

Fig. 8. PTBP1 enhanced CCND1 and MMP9 expression in a PKM2 and MEIS2-L dependent manner. a. Heat map representing unsupervised hierarchical clustering of mRNA expression levels in UM-UC-3 cells transfected with control siRNA or si-PTBP1. Each row represents the indicated sample; each column indicates one mRNA. Red and green indicate high and low expression respectively. **b, c.** Analysis of mRNA and protein expression levels of CCND1 and MMP9 after knockdown or overexpression of PTBP1 in T24 and UM-UC-3 cells. **d, f.** qRT-PCR and western blot detection of CCND1 in PTBP1-silencing or control cells, combined with overexpression of PKM2. **e, g.** qRT-PCR and western blot detection of MMP9 in PTBP1- silencing or control cells, combined with overexpression of MEIS2-L. *p < 0.05 and **p < 0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

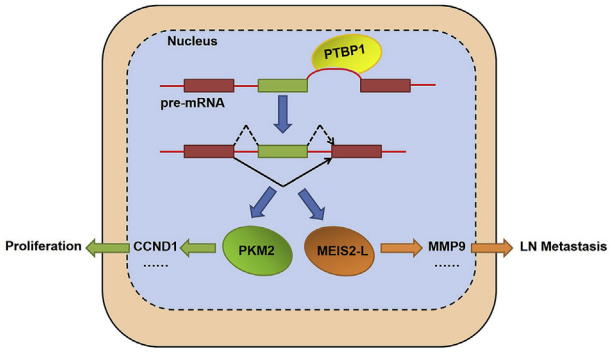

Fig. 9. A schematic model of the mechanism underlying the role of PTBP1 in bladder cancer proliferation and lymph node metastasis.

Given the important functions of alternative splicing in cancer, there is a growing need to develop therapeutic strategies to target aberrant splicing events [67]. Small molecules, such as E7107, have been designed to target the core spliceosome components, and impair cancer-related pre-mRNA splicing in a dose- and time-dependent manner while leaving other constitutive splicing events unchanged [68]. Additionally, oligonucleotide-based therapies that target specific pathologic splicing events show promise. Indeed, clinical trials for patients with Duchenne muscular dystrophy (DMD) and spinal muscular atrophy (SMA) have been established (NCT Number: NCT00844597; NCT01780246; NCT01703988; NCT01839656), investigating the modulation of splicing events as a potential disease therapy [69,70]. In the future, inhibition of cancer metastasis and proliferation via small molecules that specifically target PTBP1 and specific oncogenic splicing events might be a potential therapeutic approach for bladder cancer.

In summary, we report the novel discovery that PTBP1 clinically and functionally participates in LN metastasis and proliferation of bladder cancer through alternative splicing-mediated upregulation of MEIS2-L and PKM2 (Fig. 9). Uncovering the precise role of PTBP1 in the progression of bladder cancer will not only increase our knowledge of splicing factor-induced LN metastasis and proliferation, but will also accelerate the development of novel biomarkers and therapeutic strategies for bladder cancer patients with lymphatic metastasis.

List of abbreviations

LN, Lymph node; PTBP1, Polypyrimidine tract binding protein 1; NMIBC, non-muscle-invasive bladder cancer; MIBC, muscle-invasive

bladder cancer; AS, Alternative splicing; CNU, Chungbuk National University; hnRNP, heterogeneous nuclear ribonucleoprotein; RRM, RNA Recognition Motif; NAT, normal adjacent tissues; IHC, Immunohistochemistry; MTT, methyl thiazolyl tetrazolium; RIP, RNA immunoprecipitation; qRT-PCR, quantitative reverse-transcription PCR; MEIS2, Myeloid ecotropic insertion site 2; PKM, Pyruvate kinase isozymes; GSEA, Gene Set Enrichment Analysis; TALE, three amino-acid loop extension; HDAC3, histone deacetylase 3; DMD, Duchenne muscular dystrophy; SMA, spinal muscular atrophy.

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Conflicts of interest statement

We confirm that there are no known conflicts of interest associated with this publication.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.01.041.

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