Tohoku J. Exp. Med., 2025, **265**, 69-81

# Long Noncoding LINC00115 Facilitates Cell Growth and Inhibits Apoptosis by Regulating the miR-4701-5p/P4HB Axis in Bladder Cancer

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Bladder cancer (BCa) is a prevalent urogenital malignancy, imposing a significant burden on health-care systems worldwide. Long noncoding RNAs (lncRNAs) are important regulators of carcinogenesis and affect BCa progression. In this study, the influence of lncRNA LINC00115 on malignant behavior of BCa cells were explored. Bioinformatics method was used for prediction of gene expression and downstream molecules of LIN00115. LINC00115 expression level in BCa cells was measured using RT-qPCR. After LINC00115 depletion, the proportion of viable, proliferative, and apoptotic BCa cells were calculated by methyl thiazolyl tetrazolium (MTT) assays, colony formation assays, and TUNEL staining, respectively. FISH was performed to verify the cellular distribution of LINC00115. The interaction between LINC00115 and miR-4701-5p and the binding between miR-4701-5p and P4HB were confirmed using RNA pulldown, RNA immunoprecipitation (RIP), and luciferase reporter assays. Experimental results showed that LINC00115 was highly expressed in BCa cells. The silencing of LINC00115 restrained BCa cell proliferation and stimulated apoptosis. LINC00115 could directly bind to miR-4701-5p and thus initiate P4HB upregulation in BCa cells. P4HB 3'untranslated region could be targeted by miR-4701-5p. Additionally, Amplification of P4HB expression offset the effects of LINC00115 knockdown on BCa cell proliferative and apoptotic behaviors. In conclusion, LINC00115 facilitates BCa cell growth and inhibits apoptosis via interaction with miR-4701-5p and upregulation of P4HB.

**Keywords:** apoptosis; bladder cancer; cell proliferation; LINC00115; miR-4701-5p; P4HB
Tohoku J. Exp. Med., 2025 February, **265** (2), 69-81.
doi: 10.1620/tjem.2024.J075

## Introduction

Bladder cancer (BCa) is the 10th most prevalently diagnosed malignancy within the urinary system, becoming a global health issue (Dobruch and Oszczudłowski 2021; Dyrskjøt et al. 2023). According to GLOBOCAN data, there were 573,000 newly diagnosed BCa cases and 213,000 deaths worldwide in 2020 (Sung et al. 2021). Common clinical symptoms of BCa encompass gross painless hematuria, dysuria and upper urinary infection (Bellmunt 2015). Risk factors for BCa comprise occupational exposure, inflammation, frequent urinary catheter use, smoking and family history (Guidance N. 2017). Currently, treatments for BCa include surgery, chemotherapy, and radiotherapy, but the clinical outcomes are unsatisfactory (Aghaalikhani et al. 2019). Therefore, it is imperative to investigate molecular mechanisms underlying BCa tumorigenesis and identify biomarkers for early diagnosis.

During the past decades, noncoding RNA-based therapeutics have been demonstrated to be beneficial for the development of targeted therapies for cancer or other diseases (Kara et al. 2022). From aspects of length, location, and shape, noncoding RNA can be divided into different types, among which long ncRNA (lncRNA), microRNA

Received June 5, 2024 revised and accepted August 1, 2024; J-STAGE Advance online publication August 8, 2024
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(miRNA), circular RNA, and PIWI interacting RNA are four major ncRNA types (Wolfien et al. 2019). With no less than 200 nucleotides in length, many lncRNAs are abnormally expressed in various types of cancer and play tumor-suppressive or oncogenic roles (Jarroux et al. 2017). LncRNA-mediated competing endogenous RNA (ceRNA) networks have been known to have potential to be therapeutic targets for BCa (Li et al. 2023). According to the ceRNA hypothesis, lncRNAs bind with miRNAs, thereby modulating the regulatory role of miRNAs in the expression of protein coding genes through interaction with 3′ untranslated region (3′ UTR) of their targets (Wu et al. 2014; Alles et al. 2019; Chen et al. 2019). MiRNAs are small ncRNAs with high conservation among species and regulate gene expression by degradation of messenger RNA (mRNA) or translational inhibition (Correia de Sousa et al. 2019). Recently, the ceRNA networks mediated by lncRNAs have been widely reported (Li et al. 2023). For example, lncRNA XIST facilitates BCa cell growth, invasion, and metastasis by sponging miR-129-5p and thereby inducing upregulation of TNSF10 expression (Kong et al. 2024). LncRNA LOC339524 serves as a ceRNA for miR-875-5p and thus cause the upregulation of COPS7A, eventually obstructing BCa cell proliferation (He et al. 2021). LncRNA KCNMB2-AS1 aggravates malignant behavior of BCa cells by competing with S100A10 for the binding with miR-374a-3p (Zhu et al. 2021). According to lncRNA profiling analyses based on The Cancer Genome Atlas (TCGA) data in a previous study (Jiang et al. 2018), LINC00115 was identified to be negatively correlated to outcome of patients diagnosed with BCa using univariable Cox regression analysis. More specifically, patients with high LINC00115 expression have shorter overall survival time (Jiang et al. 2018). LINC00115 was also reported to play an oncogenic role in other types of cancer such as gastric, breast, lung, and prostate cancer by mediating various ceRNA networks (Yuan et al. 2020; Peng et al. 2021; Wu et al. 2022; Zhu et al. 2023). Nevertheless, the functions of LINC00115 in BCa and the underlying mechanism have not been explored. Therefore, this study aimed to investigate the effects of LINC00115 on malignant behavior of BCa cells and the ceRNA network mediated by LINC00115.

Based on bioinformatics analysis and experimental verification in this study, miR-4701-5p and prolyl 4-hydroxylase subunit beta (P4HB) were identified to be downstream molecules of LINC00115. Kaplan-Meier analyses (https://kmplot.com/analysis/) indicate the tumor-suppressive potential of miR-4701-5p and the oncogenic potential of P4HB in BCa. This study is the first to investigate the role of miR-4701-5p in BCa cells. P4HB has been regarded as a novel diagnostic and prognostic biomarker for BCa and promotes BCa progression (Wu et al. 2021). However, investigation of the ceRNA involving P4HB in BCa has not yet been undertaken. In this study, it was hypothesized that LINC00115 may regulate BCa cell growth via the miR-4701-5p/P4HB axis. This study has the potential to offer insights into the diagnosis and treatment of BCa.

## Material and Methods

*Cell culture*

BCa cell lines (T24, 5637 and UMUC-3) and normal urothelial cell line SV-HUC-1 were purchased from ATCC (Manassas, USA). Cells were seeded in Dulbecco's modified Eagle's medium (Gibco, Grand Island, USA) with 1% penicillin-streptomycin (Invitrogen, Carlsbad, USA) and 10% fetal bovine serum (Gibco) in an incubator (5% $CO_2$, 37°C).

*Bioinformatic methods*

LINC00115 (or P4HB) expression in bladder urothelial carcinoma (BLCA) tissue (n = 414) and normal bladder epithelium tissue (n = 19) were analyzed using UALCAN (Chandrashekar et al. 2022). The data on the UALCAN website were retrieved from TCGA database. MiRNAs that have binding site with LINC00115 were predicted with ENCORI with default screening conditions (Li et al. 2014). Targets of miR-4701 were predicted with ENCORI under the criterion of pan cancer: 4 cancer types and were listed from high to low according to the clip expectation number. Kaplan-Meier plotter analysis was performed to analyze the correlation of miR-4701 (or P4HB) expression and the survival probability of patients with BCa (n = 7,642) with default screening conditions.

*Cell transfection*

*Two* types of short hairpin RNA targeting LINC00115 (sh-LINC00115#1/2) were utilized to downregulate LINC00115. MiR-4701-5p mimics were prepared for overexpressing miR-4701-5p. Full length of LINC00115 (or P4HB) was inserted into the pcDNA3.1 vector to elevate LINC00115 (or P4HB) expression. Their corresponding plasmids are sh-NC, NC mimics, and empty pcDNA3.1 vectors. All plasmids were synthesized by GenePharma (Shanghai, China) and transfected into BCa cell lines using Lipofectamine 2000 (Invitrogen, USA) for 2 days.

*RT-qPCR*

For RNA extraction from BCa cells and reverse transcription to cDNA, TRIzol reagent (Invitrogen) and the SureScript™ First-Stand cDNA Synthesis Kit (GeneCopoeia, Guangzhou, China) were used, respectively. RT-qPCR was conducted using SYBR Green qPCR Mix (GeneCopoeia) on an Applied Biosystems (ABI) Prism 7500 Real-Time PCR System (ABI, USA). The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Nagura-Ikeda et al. 2020). GAPDH acted as an internal control for LINC00115 and mRNAs, while U6 was the control for miRNAs. The primer sequences were listed in Table 1.

*Colony formation assay*

After separated with trypsin, T24 and UMUC-3 cells

Table 1. Sequences of primers used for RT-qPCR.

| Gene | Sequence (5'→3') |
|---|---|
| LINC00115 forward | TTTAGATGGGAAGTGGATGGG |
| LINC00115 reverse | CACTCTCCTTCACTGTCGG |
| miR-588 forward | GGGUUGGCCACAAUGGGU |
| miR-588 reverse | GTCGTATCCAGTGCGTGT |
| miR-4701-5p forward | UUCCCCAUCCACACCACC |
| miR-4701-5p reverse | CUCUACAGCUAUAUUGCCAGCCAC |
| miR-212-5p forward | CGCGACCTTGGCTCTAGACTG |
| miR-212-5p reverse | AGTGCAGGGTCCGAGGTATT |
| RPS24 forward | TAGAAAGTTCATGACCAACCGA |
| RPS24 reverse | GTGAAGGACATCAATGACCA |
| P4HB forward | CTTCTTCAAGGACGTGGAG |
| P4HB reverse | ATCCCAAATGGTATGTCATCG |
| RPL23A forward | GAAAGCGAAGAAGGAAGCTC |
| RPL23A reverse | CTTTAAAGCCTTCGCTTTGG |
| GAPDH forward | CGGAGTCAACGGATTTGGTCGTAT |
| GAPDH reverse | AGCCTTCTCCATGGTGGTGAAGAC |
| U6 forward | GCTTCGGCAGCACATATACTAAAAT |
| U6 reverse | CGCTTCACGAATTTGCGTGTCAT |

were inoculated to 6-well plates (600 cells/well) for 2 weeks. When cell colonies were visible, the incubation was terminated. During the incubation, the culture medium was replaced every three days. After that, cells were fixed with paraformaldehyde (Sigma Aldrich, St. Louis, USA) for 20 min and dyed with Giemsa solution (Sigma Aldrich) for 40 min. Finally, cell colonies were manually counted under a microscope (Keyence, Japan).

*MTT assay*

After 24, 48, or 72 h of cell culture, UMUC-3 and T24 cells ($2 \times 10^3$ cells/well, 96-well) were incubated in the culture fresh medium supplemented with MTT solution (10 $\mu$L; Invitrogen) for 2 h at 37℃. Dimethyl sulfoxide (Takara, Tokyo, Japan) was utilized to dissolve the formazan and then the supernatant was discarded. Optical density values were read with a microplate reader (ELX-800, BioTek, USA) at 490 nm.

*TUNEL staining assay*

TUNEL assays were performed to detect cell apoptosis with the In Situ Cell Death Detection Kit (Takara) according to supplier's protocols. TUNEL-positive cells were shown in green. DAPI (Absin, Shanghai, China) was used for the staining of cell nuclei (in blue). Positively stained cells were calculated under EVOS FL microscope (BioTek) (Mirzayans and Murray 2020).

*Western blotting*

UMUC-3 and T24 cells were lysed in radio immunoprecipitation lysis buffer (Solarbio, Beijing, China) on ice. BCA assay kit (abcam, UK) was used to determine the protein concentration. Next, protein samples (20 $\mu$g) were fractionated using 10% SDS-polyacrylamide gel and loading to PVDF membrane (Millipore, Billerica, USA). After blocked with milk powder for 1 h, the membrane was incubated with primary antibodies of P4HB (1: 1000, ab137110, abcam), Cyclin A1 antibody (1: 1000, ab53699), Cyclin B1 (1: 1000, ab32053), Cyclin D1 (1: 1000, ab16663), CDK2 (1: 1000, ab32147) and GAPDH (1: 1000, ab181602) at 4℃ overnight. Horseradish peroxidase-labeled IgG (Abcam) was regarded as secondary antibody and incubated with cell samples at ambient temperature for another 2 h. Chemiluminescence gel imaging system (Jiapeng, Shanghai, China) was used to illuminate the blots, and ImageJ software was utilized to analyze the intensity of bands.

*Fluorescence in situ hybridization assay (FISH)*

The cellular distribution of LINC00115 in BCa cells was identified using the FISH kit (RiboBio, Guangzhou, China) following the manufacturer's recommendations. DAPI was used to stain cell nuclei. Finally, a fluorescence microscope (BioTek) was used for imaging.

*RNA pulldown assay*

LINC00115 probe with biotin (Bio-LINC00115) and negative control with biotin (Bio-NC) were obtained from Sangon (Shanghai, China). The probes were incubated with cell lysates for 1 h and then with magnetic beads (Invitrogen) overnight at 4°C. Finally, the RNA onto the magnetic beads was eluted. RT-qPCR was required for measurement of miRNA enrichment.

*Luciferase reporter assay*

The wild type or mutant type (Wt or Mut) of LINC00115 or P4HB 3′ UTR containing the binding site of miR-4701-5p was cloned into the pmirGLO vector (Promega, Madison, WI, USA) to generate LINC00115-Wt/Mut or P4HB 3′ UTR-Wt/Mut reporters. MiR-4701-5p mimics or NC mimics were co-transfected with pmirGLO reporters into BCa cells using Lipofectamine 2000. Luciferase activities of these reporters were measured with a luciferase reporter assay system (Promega).

*RNA immunoprecipitation (RIP) assay*

UMUC-3 and T24 cells detached by trypsin were collected and lysed using lysis buffer containing RNase and protease inhibitor cocktail. Cell lysates were centrifuged at 12,000 × *g* for 30 min, and then the supernatant was incubated with protein G Sepharose beads (Invitrogen) precoated with Ago2 antibody or IgG overnight at 4°C for 2 h. The beads were washed and subjected to RT-qPCR.

*Statistical analysis*

GraphPad Prism 8.0 Software (GraphPad Inc., San Diego, CA, USA) was used for data analysis. The data are expressed as the mean ± standard deviation. Student's *t*-test was used for difference comparison between two groups, and one-way analysis of variance followed by Tukey's *post hoc* test were used for significance comparison among multiple groups. A value of $p < 0.05$ was the threshold for statistical significance.

## Results

*LINC00115 depletion inhibits BCa cell proliferation and facilitates apoptosis*

According to TCGA data, LINC00115 is upregulated in BLCA tissue compared to that in normal bladder epithelium tissue (Fig. 1A, p = 1.38E-09). High LINC00115 expression was experimentally detected in three BCa cell lines (UMUC-3, T24, and 5637) relative to that in the normal urothelial cell line SV-HUC-1 using RT-qPCR (Fig. 1B, p < 0.001). In addition, UMUC-3 cell line and T24 cell line displayed the most significantly increased LINC00115 expression (6.7 folds and 6.1 folds) compared with the other cell line 5637 (5.4 folds) (Fig. 1B). Therefore, UMUC-3 and T24 cells were chosen for following functional experiments, while the cell line 5637 was excluded from most of the subsequent experiments. LINC00115 level was downregulated in BCa cells transfected with sh-LINC00115 plasmids in comparison to its expression in the sh-NC group (Fig. 1C, p < 0.001). MTT and colony forming assays demonstrated that LINC00115 deficiency significantly repressed BCa cell viability and proliferation by reducing optical density (OD) value and the number of colonies, respectively (Fig. 1D, E). For CCK-8 assays, the OD value of UMUC-3 cells at 72 h was lowered to 0.61 and 0.58 by two types of sh-LINC00115 plasmids, and that of T24 cells was reduced to 0.65 and 0.62 compared with the OD value in the sh-NC groups (UMUC-3: 1, T24: 1.08) (Fig.1D). The number of UMUC-3 cell colonies was reduced from 135 (sh-NC group) to 23 and 20 (sh-LINC00115#1 and #2), and that of T24 cell colonies was lowered from 117 (sh-NC group) to 25 and 24 (sh-LINC00115#1 and #2) (Fig. 1E). Moreover, TUNEL assay revealed that LINC00115 silencing facilitated BCa cell apoptosis, as evidenced by higher apoptotic rates in sh-LINC00115 groups (UMUC-3: 24.15% and 25.66%; T24: 24.85% and 24.05%) relative to that in the control (UMUC-3: 6.57% and T24: 7.24%) (Fig. 1F, G). Furthermore, depletion of LINC00115 led to a decrease in protein levels of cell cycle markers (Cyclin-A1, -B1, -D1, and CDK2) in BCa cells (Fig. 1H, p < 0.001). Overall, knockdown of LINC00115 accelerates BCa cell apoptosis while inhibiting cell proliferation and cell cycle progression.

*LINC00115 upregulation promotes BCa cell growth*

Afterwards, gain-of-function experiments were performed to measure the effects of overexpressed LINC00115 on BCa cell proliferation, apoptosis, and cell cycle progression. As shown by Fig. 2A, LINC00115 expression was prominently increased in BCa cells after transfection of pcDNA-LINC00115 vectors compared to its expression in control vector group. Cell viability was significantly higher in the pcDNA-LINC00115 group than the control vector group (Fig. 2B, *p < 0.05). Consistently, results of colony formation assays verified the promoting effect of LINC00115 upregulation on cell proliferation, as evidenced by more cell colonies in the pcDNA-LINC00115 groups (186 and 192) than the control vector groups (124 and 128) (Fig. 2C). On the contrary, overexpression of LINC00115 led to the diminished number of TUNEL-positive cells (UMUC-3: 2.15%; T24: 3.12%) compared with the control vector groups (6.72% and 7.85%), suggesting the inhibitory effect of LINC00115 on BCa cell apoptosis (Fig. 2D). Moreover, protein levels of cell cycle markers (Cyclin A1, Cyclin B1, Cyclin D1, and CDK2) were all increased in response to upregulation of LINC00115 in contrast to their protein expression in the control vector group (Fig. 2E, p < 0.001). Findings in this subsection demonstrated that LINC00115 can directly promote BCa cell proliferation and cell cycle progression while repressing cell apoptosis, which were opposite to results of loss-of-function experiments using sh-LINC00115 cell groups.

*LINC00115 binds to miR-4701-5p*

Results of FISH revealed that LINC00115 was mainly detected in cytoplasm of BCa cells (green) (Fig. 3A), suggesting the regulatory role of LINC00115 at the post-transcriptional level. To investigate the ceRNA network mediated by LINC00115, miRNAs that can bind to LINC00115 were predicted using bioinformatics methods, and 3 candidate miRNAs (miR-588, miR-4701-5p, and miR-212-5p) were identified (Fig. 3B). According to RNA pulldown experiments, miR-4701-5p had a significantly higher enrichment in the Bio-LINC00115 group in contrast to that

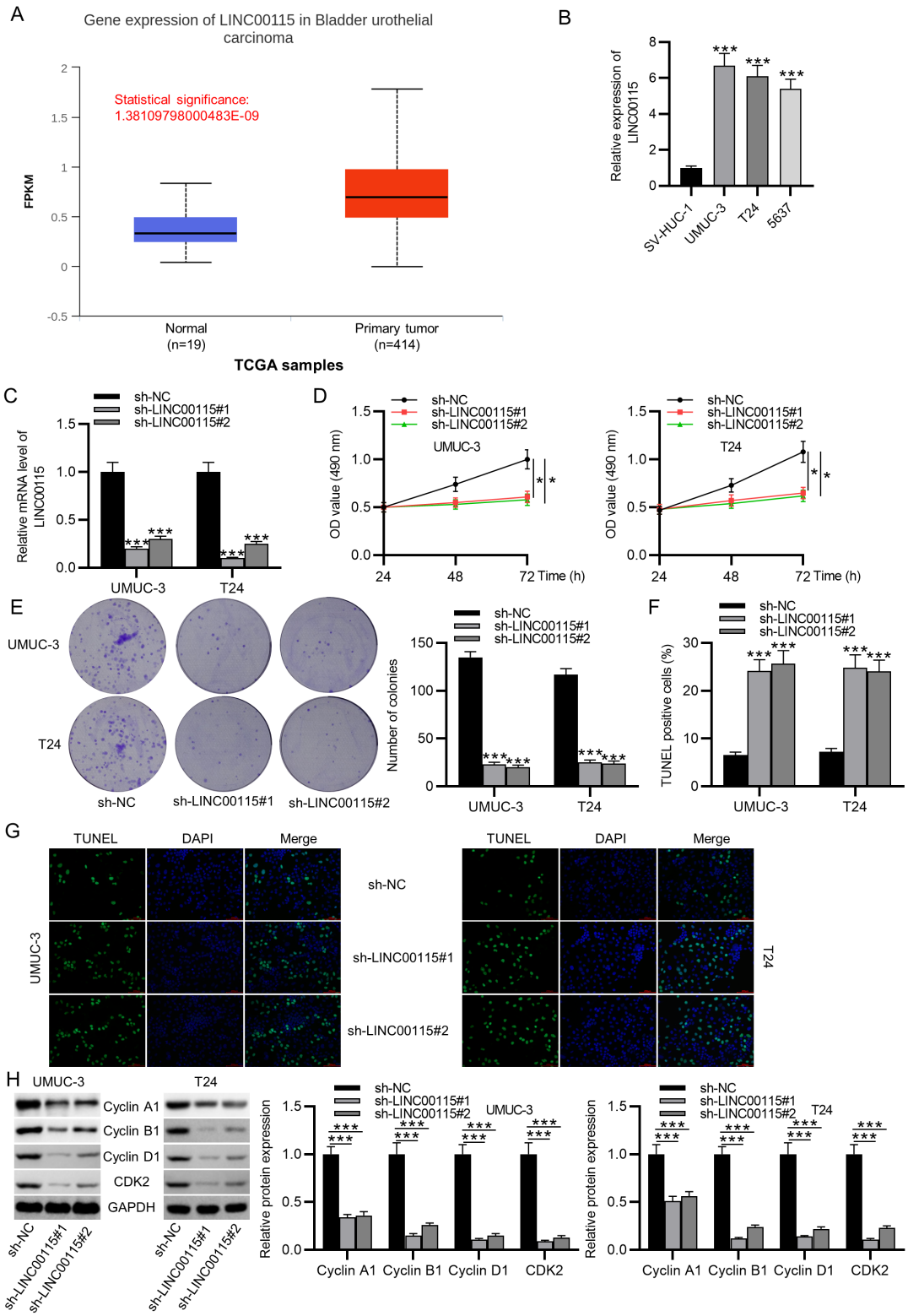


Fig. 1. LINC00115 depletion inhibits BCa cell proliferation and facilitates apoptosis.
A: LINC00115 expression in BLCA samples and normal samples was predicted by UALCAN (https://ualcan.path.uab.edu). B: RT-qPCR was performed to measure LINC00115 expression in BCa cell lines and normal urothelial cell line SV-HUC-1. C: RT-qPCR was conducted for measurement of LINC00115 expression in BCa cells transfected with sh-LINC00115#1/2. D, E: MTT and colony formation assays were carried out to evaluate BCa cell viability and proliferation after LINC00115 inhibition. F, G: TUNEL staining assays were conducted for detection of cell apoptosis after LINC00115 downregulation. H: Protein levels of Cyclin A1, Cyclin B1, Cyclin D1 and CDK2 in BCa cells after LINC00115 depletion were quantified by western blotting. *p < 0.05, ***p < 0.001.

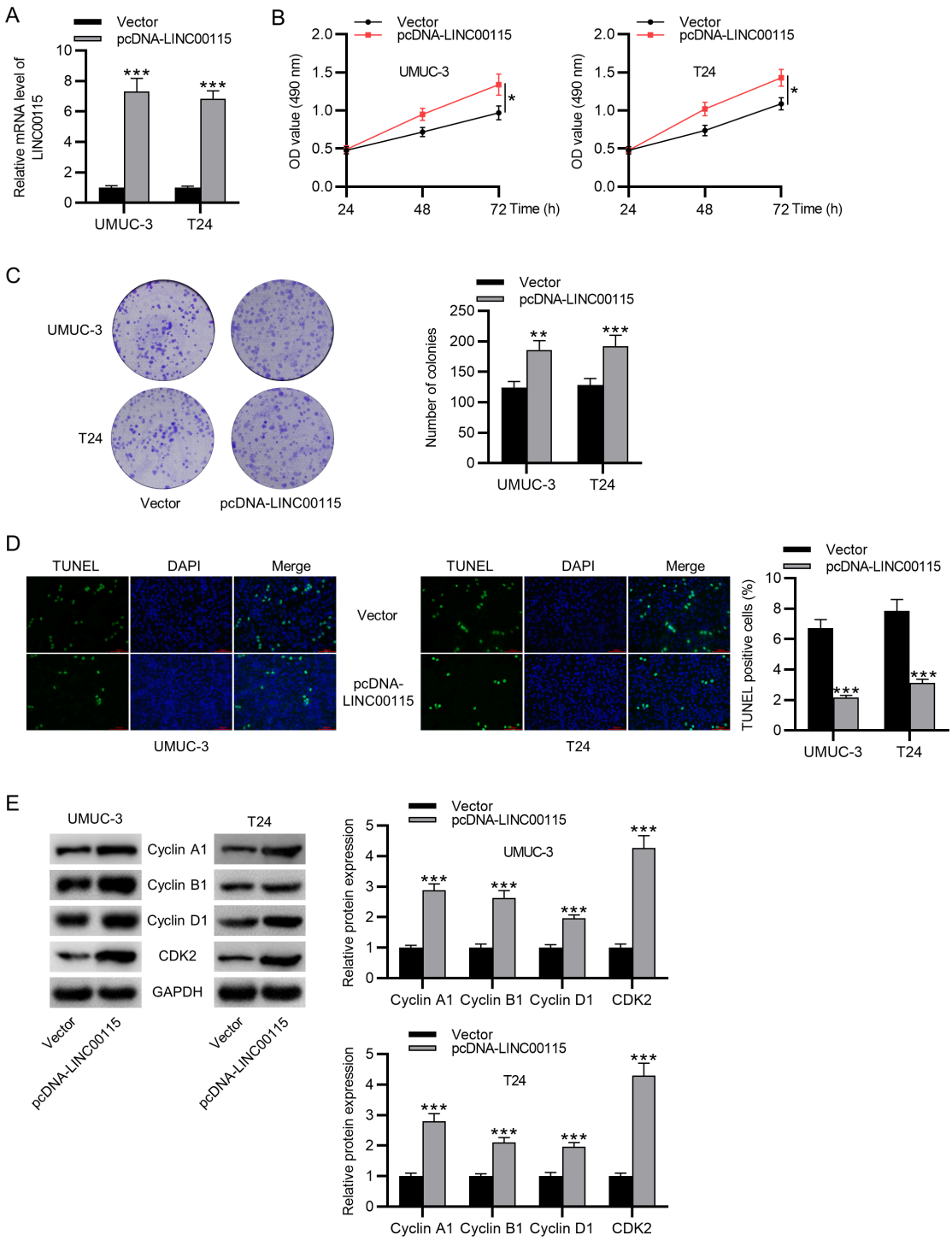


Fig. 2. LINC00115 upregulation promotes BCa cell growth.

A: RT-qPCR was performed to measure the overexpression efficacy of LINC00115 after transfection of pcDNA-LINC00115 vectors or control vectors. B, C: BCa cell viability and proliferation in the context of LINC00115 overexpression were measured using MTT and colony formation assays by calculating optical density and the number of colonies. D: TUNEL staining was conducted to determine the proportion of TUNEL positive cells (cell apoptotic rate) in pcDNA-LINC00115 group or control vector group. E: Western blotting was conducted to measure the impact of LINC00115 upregulation on protein levels of Cyclin A1, Cyclin B1, Cyclin D1, and CDK2. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

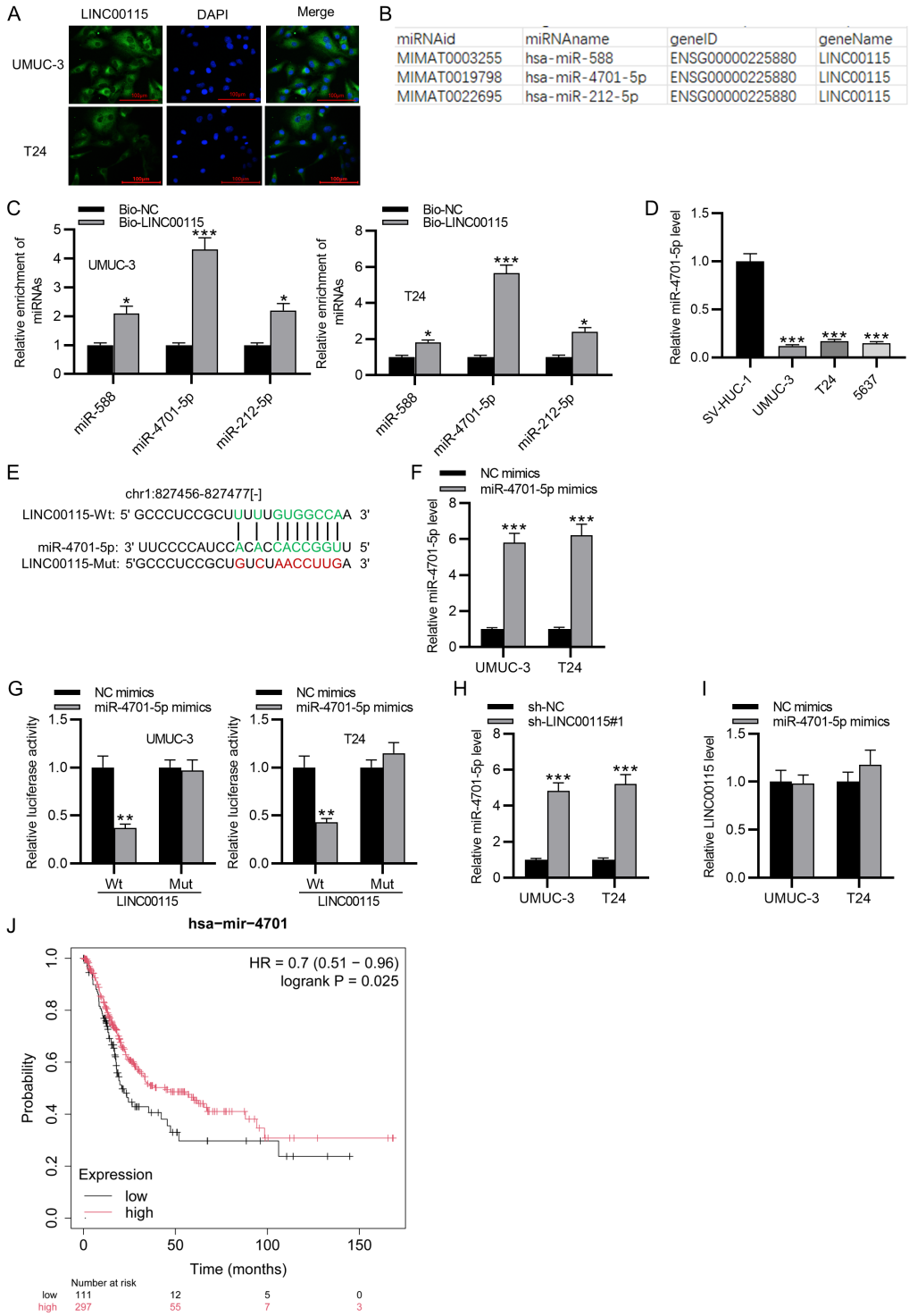


Fig. 3. LINC00115 binds to miR-4701-5p.

A: FISH assay was performed to identify the cellular localization of LINC00115 in BCa cells. B: Potential miRNAs having binding sites for LINC00115 were predicted using ENCORI. C: RNA pulldown assays was performed to assess the enrichment of candidate miRNAs pulled down by the Bio-LINC00115 probe. D: RT-qPCR was performed to measure miR-4701-5p level in BCa cell lines and normal urothelial cell line SV-HUC-1. E: A binding site between LINC00115 and miR-4701-5p was predicted using ENCORI. F: MiR-4701-5p expression in UMUC-3 and T24 cells transfected with miR-4701-5p mimics or NC mimics was measured by RT-qPCR. G: Luciferase reporter assay was performed to verify the interaction between LINC00115 and miR-4701-5p in BCa cells. H, I: The effect of LINC00115 deficiency on miR-4701-5p expression (H) and the impact of miR-4701-5p overexpression on LINC00115 expression (I) were measured by RT-qPCR. J: Kaplan-Meier plotter analysis was performed to measure the correlation between miR-4701 expression and the prognosis of patients. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

in the Bio-NC group (Fig. 3C, $p < 0.001$). Therefore, the other 2 miRNAs with less abundant enrichment in the Bio-LINC00115 group were excluded from following experiments. MiR-4701-5p was detected to be downregulated in BCa cells relative to that in normal epithelial cell line using RT-qPCR (Fig. 3D, $p < 0.001$). A binding site between LINC00115 and miR-4701-5p (chr1:827456-827477) predicted by ENCORI is shown in Fig. 3E. Luciferase reporter assays were performed to assess the binding between the two factors by measuring the effect of miR-4701-5p overexpression on the activity of luciferase reporters. The overexpression efficacy of miR-4701-5p after transfection of miR-4701-5p mimics was measured using RT-qPCR. As shown by Fig.3F, miR-4701-5p expression was markedly elevated in UMUC-3 cells and T24 cells of miR-4701-5p mimics group compared to that in the NC mimics group (5.8 folds and 6.22 folds) (Fig. 3F). Overexpression of miR-4701-5p significantly diminished the luciferase activity of LINC00115-Wt in UMUC-3 cells (63% decrease) and T24 cells (57% decrease) while exerting no significant effect on miR-4701-5p-Mut activity, as revealed by luciferase reporter assay (Fig. 3G). Moreover, PCR revealed that LINC00115 depletion led to a higher miR-4701-5p level in BCa cells (Fig. 3H, $p < 0.001$), while miR-4701-5p overexpression did not significantly alter the expression of its upstream factor LINC00115 (Fig. 3I). The data suggest that LINC00115 inversely regulates miR-4701-5p level in BCa cells. Kaplan-Meier plotter analysis showed that high miR-4701 levels correlate to better outcome of patients with BCa (Fig. 3J, $p = 0.025$), confirming its tumor suppressive role.

*MiR-4701-5p targets P4HB*

MiR-4701-5p targets were predicted using ENCORI and were listed according to the clip expectation number. Information of the first three genes were listed with the clip expectation number of 30, 15, and 14 (Fig. 4A). Fig. 4B, C revealed that miR-4701-5p overexpression prominently reduced mRNA expression of P4HB ($p < 0.001$) and did not significantly change the expression of the other two genes in BCa cells, suggesting that P4HB can be inversely regulated by miR-4701-5p. Hence, P4HB was identified for further exploration. The binding area between P4HB 3'UTR and miR-4701-5p (chr17:81843601-81843629) is predicted with ENCORI (Fig. 4D). MiR-4701-5p mimics noticeably reduced P4HB-Wt activity ($p < 0.01$) instead of P4HB-Mut activity (Fig. 4E). Additionally, LINC00115, miR-4701-5p and P4HB were all massively enriched in the Ago2 group compared with their enrichment in the IgG group ($p < 0.001$), suggesting that the 3 molecules coexist in the RNA-induced silencing complex (Fig. 4F).

*LINC00115 positively regulates P4HB expression via miR-4701-5p*

RT-qPCR and western blotting were carried out to examine the regulatory effects of LINC00115 or miR-4701-5p on P4HB expression. As illustrated by Fig. 5A, LINC00115 silencing contributed to a decrease in P4HB mRNA and protein levels in BCa cells. Overexpressed miR-4701-5p markedly reduced P4HB protein expression (Fig. 5B). The data demonstrated that P4HB expression was positively regulated by LINC00115 while being negatively regulated by miR-4701-5p in BCa cells. PCR analysis showed the high P4HB expression in BCa cells (Fig. 5C), which is consistent with the TCGA data showing higher P4HB level in primary BLCA samples than normal bladder epithelium samples (Fig. 5D, $p = 3.90E\text{-}12$). According to Kaplan-Meier plotter analysis, high P4HB expression correlates to poor outcome of patients with BCa (Fig. 5E). In conclusion, P4HB plays an oncogenic role in BCa and is indirectly, positively regulated by LINC00115 via miR-4701-5p.

*LINC00115 inhibition obstructs BCa cell proliferation and cell cycle progression while promoting apoptosis via downregulation of P4HB*

Subsequent experiments were conducted to investigate whether P4HB is responsible for alterations in proliferation and apoptosis in response to LINC00115 knockdown. P4HB was successfully overexpressed in BCa cells post pcDNA3.1/P4HB transfection in comparison to its expression in control vectors (Fig. 6A, $p < 0.001$). P4HB upregulation counteracted the repressive influence of LINC00115 deficiency on BCa cell viability and proliferative capacity, as evidenced by improved OD values and number of colonies in the sh-LINC00115#1 + pcDNA3.1/P4HB group compared with the sole sh-LINC00115#1 group (Fig. 6B, C, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). In addition, overexpressed P4HB reversed ($p < 0.01$) the high apoptosis rate induced by LINC00115 depletion ($p < 0.001$) in BCa cells (Fig. 6D). Furthermore, overexpression of P4HB rescued the decreased levels of cell cycle markers in the context of LINC00115 knockdown in BCa cells (Fig. 6E, $p < 0.001$). To sum up, overexpression of P4HB mitigates the inhibitory effects of LINC00115 depletion on malignant cellular behavior in BCa. The findings implied that LINC00115 promotes BCa cell proliferation and inhibits cell apoptosis via P4HB upregulation.

## Discussion

LncRNAs are gaining increasing significance due to their involvement in the cancer pathway and their ability to participate in tumor-suppressive or carcinogenic pathways (Mehmandar-Oskuie et al. 2023). In this study, LINC00115 was demonstrated to interact with miR-4701-5p and thus upregulate P4HB level, thereby facilitating the malignant phenotypes of BCa cells.

This study verified the upregulation of LINC00115 in BCa cells. Importantly, LINC00115 knockdown inhibited BCa cell proliferation while facilitating apoptosis, and LINC00115 overexpression exerted the opposite effect on BCa cell growth. Likewise, the oncogenic function of LINC00115 has been found in other cancer cells. For

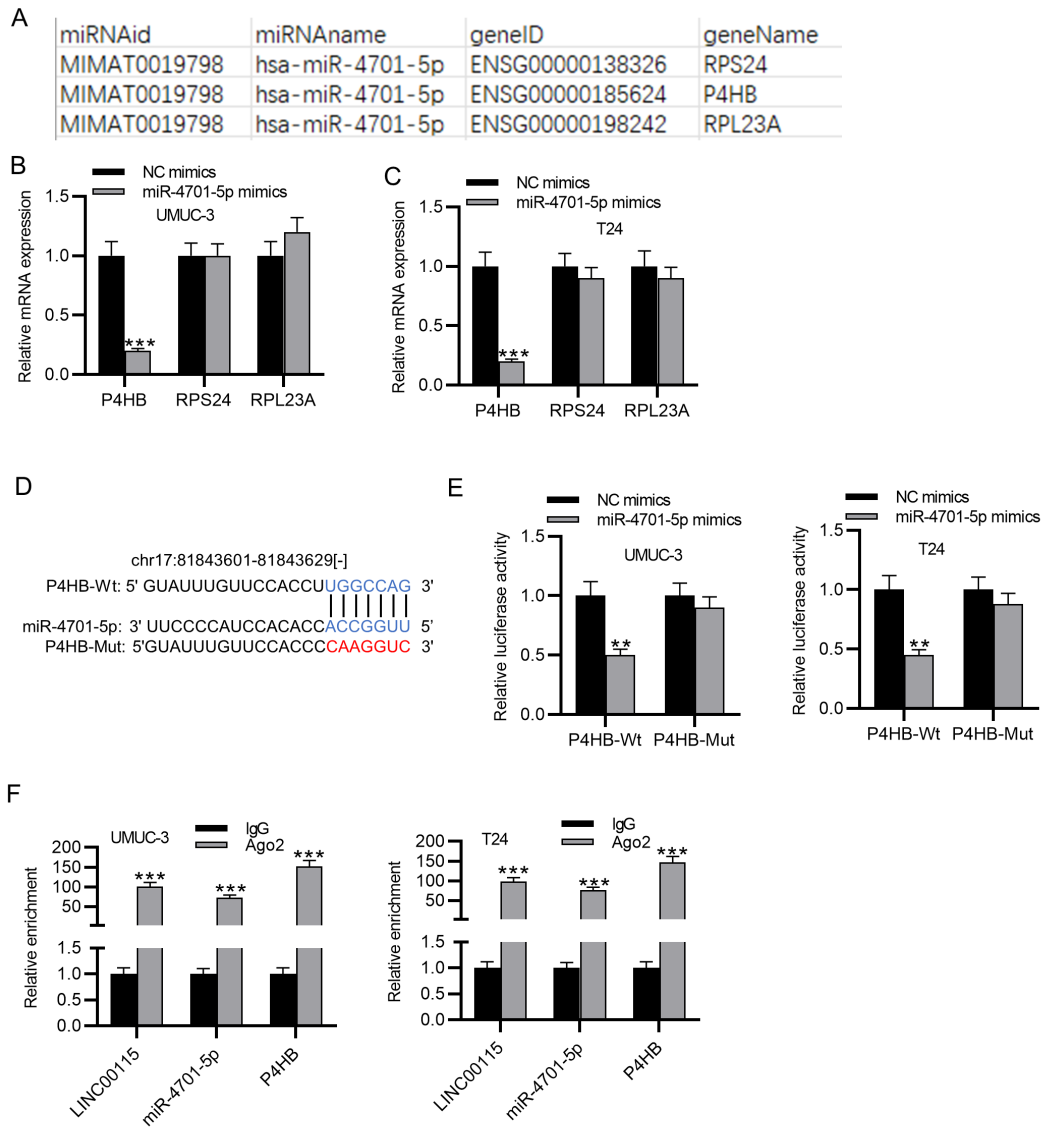


Fig. 4. MiR-4701-5p targets P4HB.
A: Possible mRNAs of miR-4701-5p were predicted using ENCORI. B, C: RT-qPCR was used to assess expression levels of candidate genes in BCa cells overexpressing miR-4701-5p. D: A binding site between miR-4701-5p and P4HB was predicted using ENCORI. E: Luciferase reporter assay was conducted to validate the interaction between miR-4701-5p and P4HB in BCa cells. F: RIP assay was performed to verify the coexistence of LINC00115, miR-4701-5p and P4HB in RNA-induced silencing complex. $^{**}p < 0.01$, $^{***}p < 0.001$.

example, the silencing of LINC00115 hampered lung cancer cell proliferation and invasion (Shao et al. 2021). LINC00115 aggravates stemness of ovarian cancer stem cells while inhibiting apoptosis via inhibition of Wnt/beta-catenin pathway (Hou and Jiang 2021). LINC00115 contributes to the carcinogenesis of thyroid cancer via regulation of the miR-489-3p/EVA1A axis and Hippo signaling (Cao et al. 2024).

As to the mechanism underlying the oncogenic role of LINC00115, it was discovered in this study that the majority of LINC00115 was in the cytoplasm of BCa cells. The finding showed the post-transcriptional regulatory potential of LINC00115 as a ceRNA in BCa. Moreover, miR-4701-5p was confirmed to bind to LINC00115 and was negatively modulated by LINC00115 in BCa cells. The study first reported the downregulation of miR-4701-5p expression in BCa cells, indicating its anti-tumor potential in BCa. Based on bioinformatics results from Kaplan-Meier plotter, a low miR-4701 level correlates to poor prognosis of patients with BCa, which further confirmed the current findings. Previously, miR-4701-5p was reported to be sponged by circular RNA circ_0065378, and miR-4701-5p acts as a tumor-promotor in colorectal cancer (Yan et al. 2022), which is different from its tumor-suppressive

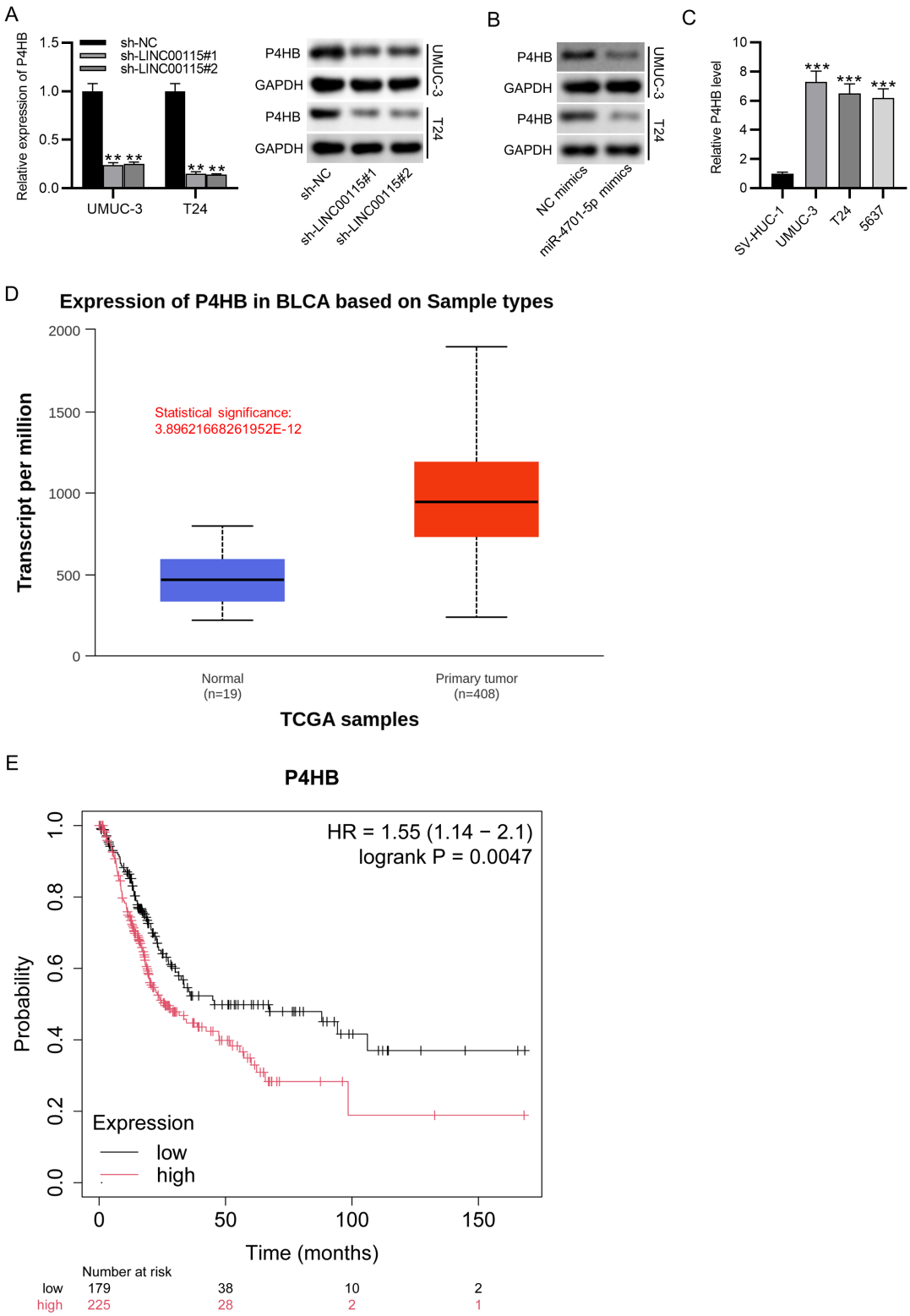


Fig. 5. LINC00115 positively regulates P4HB expression via miR-4701-5p.
A: RT-qPCR and western blotting were conducted to evaluate P4HB mRNA and protein expression after silencing LINC00115. B: Western blotting was used to quantify P4HB protein expression in BCa cells overexpressing miR-4701-5p. C: RT-qPCR was used to detect P4HB level in BCa cell lines and SV-HUC-1 cells. D: P4HB expression in BLCA samples and normal bladder epithelium samples was predicted by UALCAN. E: The correlation between P4HB expression and the survival of patients was shown using Kaplan-Meier plotter. **p < 0.01, ***p < 0.001.

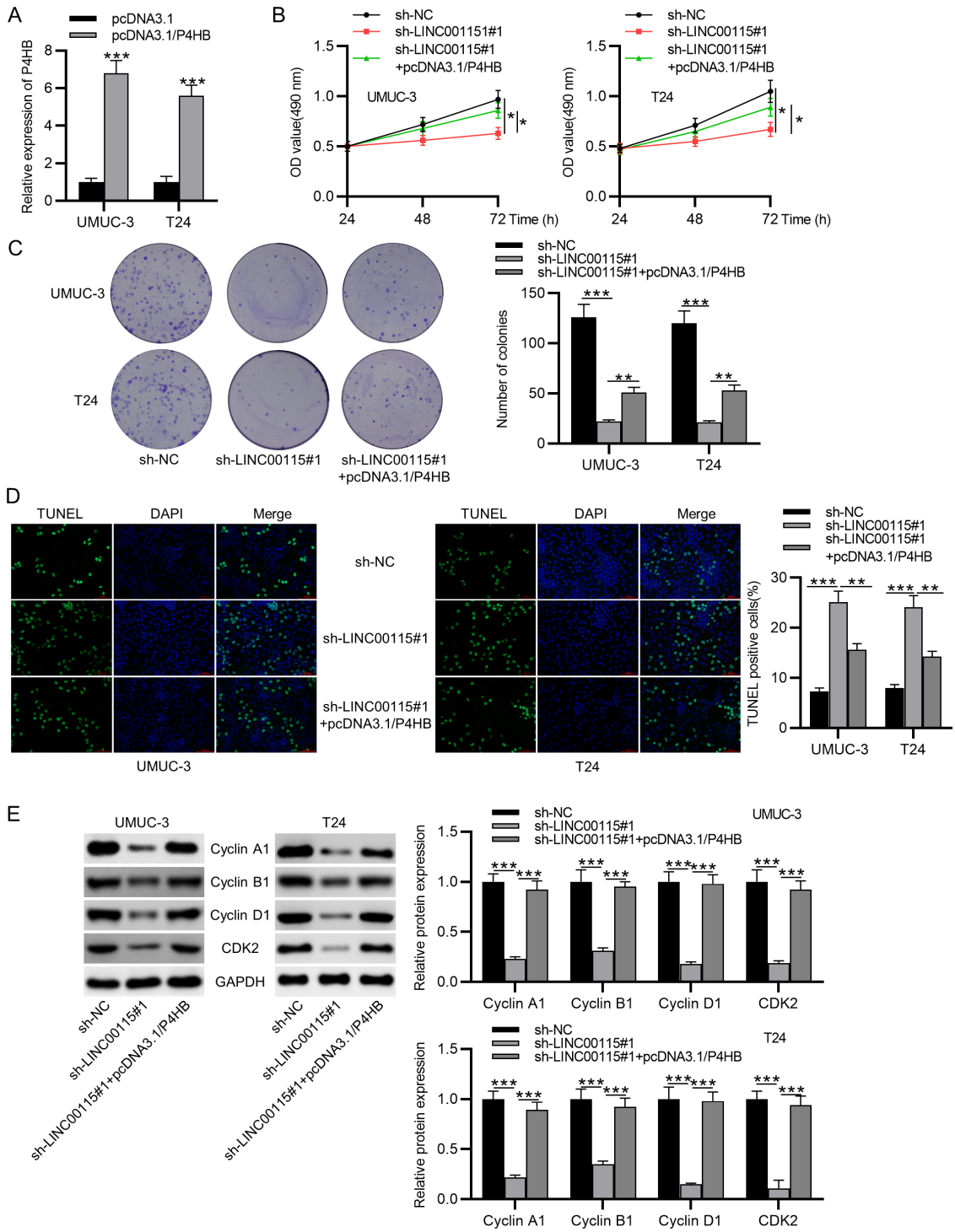


Fig. 6. P4HB overexpression attenuates the suppressive effects of LINC00115 inhibition on malignant cellular behaviors in BCa.

A: P4HB expression in BCa cells transfected with pcDNA3.1/P4HB vectors or control vectors was measured using RT-qPCR. B, C: MTT and colony formation assays were carried out to assess BCa cell viability and proliferation in sh-NC, sh-LINC00115#1 or sh-LINC00115#1 + pcDNA3.1/P4HB groups. D: Through TUNEL staining assay, the apoptosis of BCa cells in the context of LINC00115 deficiency and P4HB overexpression was measured. E: Western blotting was performed to quantify protein levels of cell cycle markers (Cyclin A1, Cyclin B1, Cyclin D1 and CDK2) in BCa cells silencing LINC00115 and overexpressing P4HB. *$p < 0.05$, **$p < 0.01$. ***$p < 0.001$.

role in the present work. MiR-4701-5p is lowly expressed in chemo-sensitive chronic myeloid leukemia cells in comparison to that in chemo-resistant cells (Li et al. 2016). Overall, the role of miR-4701-5p in cancer has not been widely reported, and its biological functions require to be further investigated in the future.

P4HB is the target gene of miR-4701-5p and is involved in the ceRNA network mediated by LINC00115 in this study. P4HB is an endoplasmic reticulum molecular chaperone protein and is reported to be oncogenic in various tumor types and involved in diverse regulatory mechanisms. For example, the silencing of P4HB causes colon cancer HT29 cell apoptosis by promoting reactive oxygen species and inactivating the STAT3 signaling pathway (Zhou et al. 2019). P4HB promotes the viability and epithelial-mesenchymal transition of adriamycin-resistant liver cancer cells by activating $\beta$-catenin/snail pathway (Ma et al. 2020). Recently, P4HB has been reported to have prognostic value in BCa, and high P4HB expression can affect overall or recurrence-free survival of patients with BCa (Wu et al. 2021). P4HB knockdown facilitates the sensitivity of BCa cells to gemcitabine by increasing the apoptotic rate and inducing cell cycle arrest at G2/M phrase (Wang et al. 2020). Inhibition of P4HB contributes to reduced proliferation ability, increased apoptotic rate, and diminished migration and invasion ability of BCa cells (T24 and 5637) (Zou et al. 2023). In line with these previous articles, the current work demonstrated that P4HB overexpression rescued the suppressive effects of LINC00115 depletion on BCa cell proliferation and cell cycle progression while reversing the promoting impact of sh-LINC00115 on cell apoptosis. The findings indicate the oncogenic role in BCa cells by promoting cell proliferation and cell cycle progression while inhibiting cell apoptosis, and LINC00115 contributes to malignant behavior of BCa cells by upregulating P4HB. Moreover, the binding of miR-4701-5p to P4HB was first validated in the present work, and P4HB level was positively regulated by LINC00115 via miR-4701-5p.

Collectively, LINC00115 binds to miR-4701-5p and indirectly upregulates P4HB to facilitate BCa cell proliferation and suppress apoptosis. The findings may provide a promising therapeutic approach for BCa treatment. However, animal experiments were not conducted to further explore the role of LINC00115/miR-4701-5p/P4HB axis in BCa tumorigenesis. Additionally, other regulatory factors or signaling pathways related to the axis in BCa need to be further investigated. Therefore, more experiments should be conducted in our future work to elucidate more comprehensive and intricate strategies for BCa treatment.

## Author Contributions

Changyuan Dai was the main designer of this study. Changyuan Dai, Qingwen Li, Lili Wang, Jiajun Zhang and Shuai Yang analyzed the data. Changyuan Dai, Qingwen Li and Xiaole Zhang drafted the manuscript. All authors read and approved the final manuscript.

## Funding

Bengbu Science and Technology Innovation Guidance Project: Expression and mechanism of action of miR-15a in prostate cancer cell models (Project number: 20200348). Natural Science Key Fund of Bengbu Medical College (Project number: 2023byzd051).

## Conflict of Interest

The authors declare no conflict of interest.

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