Article

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PTBP1 Enhances Epithelial-Mesenchymal Transition by Upregulating *HOXA9* to Drive Pancreatic Cancer Progression

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Background: Polypyrimidine tract binding protein 1 (PTBP1) plays a crucial role in the stemness of various cancer types. Therefore, this study aimed to unveil the potential role of PTBP1 in the metastatic progression of pancreatic cancer (PC).
Methods: PTBP1 expression was confirmed in PC using bioinformatics analysis and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) data. After correlation analysis, RNA immunoprecipitation assay was employed to assess the interaction between PTBP1 and homeobox A9 (*HOXA9*). Based on the gain- or loss-of-function of the gene, the impact of PTBP1 in PC cells and its underlying regulatory mechanism in malignant behavior were investigated using a cell counting kit-8 (CCK-8) assay, flow cytometry, a Transwell assay and Western blot analysis.
Results: PTBP1 was found to be upregulated in PC. PTBP1 silencing enhanced cell apoptosis and dysregulated cell viability, migration, and invasion. PTBP1 silencing repressed epithelial-mesenchymal transition (EMT) by upregulating epithelial cadherin (E-cadherin) and downregulating neural cadherin (N-cadherin), Vimentin, and Snail. However, these effects were reversed by the overexpression of *HOXA9*. PTBP1–*HOXA9* interaction upregulated *HOXA9* expression. Furthermore, *HOXA9* overexpression reduced apoptosis and enhanced viability, migration, invasion, and EMT in PC cells. However, these effects were counteracted by the knockdown of PTBP1.
Conclusions: PTBP1 drives EMT and promotes PC cell survival, migration, and invasion through interacting and upregulating *HOXA9* expression.

Keywords: pancreatic cancer; polypyrimidine tract binding protein 1; homeobox A9; epithelial-mesenchymal transition

Introduction

Pancreatic cancer (PC), primarily pancreatic adenocarcinoma (PAAD), is a highly lethal clinical condition, with a median survival of approximately 4 months and a 5-year survival of 13% [1,2]. Due to a dearth of effective early diagnosis and the generally poor prognosis, improving patient's outcomes would rely on approaches that alleviate their invasive and metastatic phenotype, increase sensitivity to adjuvant therapies, and enhance surgical resection rates through molecular-biology guided strategies.

Epithelial-mesenchymal transition (EMT) is a complex and multi-step cellular program driven by relevant transcription factors such as Snail and Twist that alter cell-cell adhesion and apical-basal cell polarity while inducing mesenchymal characteristics [3]. In addition to its essential roles in embryogenesis, wound healing and fibrogenesis, EMT is closely linked to tumor progression [4]. Recent evidence has demonstrated that EMT provides cancer cells with plasticity and stemness that drive tumorigenesis, metastasis, and acquired drug resistance [5–7]. A study by Wei *et al.* [8] reported that the overexpression of Snail promotes EMT and stem-like characteristics of PC cells, thereby enhancing gemcitabine resistance *in vivo*. Therefore, deciphering the molecular mechanisms underpinning EMT in PC is crucial for identifying novel therapeutic targets to alleviate malignant progression.

Based on cancer single-cell functional analysis using the CancerSEA database (http://biocc.hrbmu.edu.cn/CancerSEA/), we observed a correlation between polypyrimidine tract binding protein 1 (PTBP1) and EMT. PTBP1 is an RNA-binding protein ubiquitously expressed across human tissues. Evidence reveals that PTBP1 regulates the expression of post-transcriptional genes and is involved in several cellular processes, including embryonic development, neuronal cell differentiation, and T-cell activation [9]. Dysregulated PTBP1 expression has been increasingly reported in various diseases, particularly cancers [10–12]. In gastric cancer, increased PTBP1 is linked to poor overall survival, and its overexpression facilitates cell proliferation and migration, probably by inducing a stem-like state [13].

Therefore, we systematically explored the influence of PTBP1 on PC metastatic progression *in vitro* and investigated its underlying mechanisms.

Materials and Methods

Bioinformatics Analysis

PTBP1 expression in PAAD was analyzed using the Gene Expression Profiling Interactive Analysis (GEPIA) platform (http://gepia2.cancer-pku.cn/#index) with The Cancer Genome Atlas (TCGA) data (179 tumor and 171 normal samples). The correlation between *PTBP1* and homeobox A9 (*HOXA9*) expressions was assessed across 178 PAAD samples utilizing the Encyclopedia of RNA Interactomes (ENCORI) platform (https://rnasysu.com/encori/).

Cell Culture

Human pancreatic duct epithelial cell line (HPDE6-C7; H1-3201, Cyagen Biosciences, Guangzhou, China) and PC cell lines SW1990 (CL-0448, Procell, Wuhan, China) as well as PANC-1 (SCSP-535, Cell Bank of Shanghai Academy of Chinese Sciences, Shanghai, China) were used in this study. PANC-1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; BasMed-AW-001, AnWei-sci, Shanghai, China) containing 1% L-glutamine (C0212, Beyotime, Shanghai, China), 15% fetal calf serum (FBS; S4115, Biochrom AG, Berlin, Germany), and 1% penicillin-streptomycin (P/S; C0222, Beyotime, Shanghai, China). HPDE6-C7 and SW1990 cells were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin. All cell cultures were maintained and passaged at 37 °C in a humidified condition of 5% CO_2, examined for mycoplasma contamination, and authenticated by short tandem repeat (STR) profiling.

RNA Immunoprecipitation (RIP) Assay

PTBP1–*HOXA9* interaction in PC cells was assessed using the Magna RIP kit (17-700, Sigma-Aldrich, St. Louis, MO, USA). Briefly, 1×10^7 cells were washed with ice-cold phosphate-buffered saline (PBS; R24000, Yuanye Bio-Technology Co., Ltd., Shanghai, China) and then centrifuged. The resultant cell pellets were homogenized on ice for 5 minutes in RIP lysis buffer containing protease inhibitor cocktail and ribonuclease (RNase) inhibitor. Meanwhile, A/G magnetic beads were pre-labeled with anti-PTBP1 antibody (32-4800, Thermo Fisher, Waltham, MA, USA) or control IgG (36111ES10, Yeasen, Shanghai, China) at room temperature for 30 minutes, and subsequently immunoprecipitated with the lysates overnight at 4 °C. After that, immune complexes were eluted using a magnetic separator and treated with proteinase K buffer to purify RNA. Finally, immunoprecipitated RNA was analyzed using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR).

PTBP1 Silencing and HOXA9 Overexpression

Short hairpin RNA targeting *PTBP1* (shPTBP1; sense: 5′-GCTCCAAGAACTTTCAGAACA-3′, antisense: 5′-TGTTCTGAAAGTTCTTGGAGC-3′) and a negative control (shNC; C02001) were obtained from GenePharma (Shanghai, China). An *HOXA9* overexpression vector (PPL02064-4b) and an empty vector negative control (NC) were purchased from the Public Protein/Plasmid Library (Nanjing, China). The *HOXA9* coding sequence is given in **Supplementary File 1**. PC cells in logarithmic growth were harvested and reseeded in 96-well plates. Transfections with shPTBP1 or the *HOXA9* expression plasmids were conducted at 37 °C using Lipofectamine 3000 reagent (L3000075, Thermo Fisher, Waltham, MA, USA), following the manufacturer's instructions.

RNA Extraction and qRT-PCR

Total RNA was isolated from the immunoprecipitated complexes or PC cells using the Trizol method (15596026, Thermo Fisher, Waltham, MA, USA) and was subsequently reverse transcribed to complementary DNA (cDNA) using the AdvanceFast First Strand cDNA Synthesis Kit (11149ES10, Yeasen, Shanghai, China). Then qRT-PCR was conducted with gene-specific primers using 2× Hieff PCR Master Mix (10102ES08, Yeasen, Shanghai, China) on a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). *PTBP1* and *HOXA9* mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to an internal reference glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). For group comparisons, expression was normalized to HPDE6-C7 cells in the corresponding control (Control or NC+shNC group). Primers used for cDNA amplification are listed as follows (5′-3′): *PTBP1* (forward (F): AGCGCGTGAAGATCCTGTTC, reverse (R): CAGGGGTGAGTTGCCGTAG), *HOXA9* (F: TACGTGGACTCGTTCCTGCT, R: CGTCGCCTTGGACTGGAAG), epithelial cadherin (*E-cadherin*) (F: CTGTGCCCAGCCTCCATGTTTT, R: CTGGATAGCTGCCCATTGCAAGTTA); neural cadherin (*N-cadherin*) (F: TTATCCTTGTGCTGATGTTTGTG, R: TCTTCTTCTCCTCCACCTTCTTC); *Vimentin* (F: TGTCCAAATCGATGTGGATGTTTC, R: TTGTACCATTCTTCTGCCTCCTG); *Snail* (F: CAATCGGAAGCCTAACTA, R: CAGATGAGCATTGGCAGCG) and *GAPDH* (F: ACAACTTTGGTATCGTGGAAGG, R: GCCATCACGCCACAGTTTC).

Cell Counting Kit-8 (CCK-8) Assay

PC cells were seeded into 96-well plates at a density of 5×10^3 cells/well, and were subsequently transfected with *PTBP1* silencing and/or *HOXA9* overexpression vectors. Cell viability was evaluated using the CCK-8 assay (E1008, Applygen Technologies, Beijing, China); 10 µL reagent was added to each well and incubated at 37 °C for 4 hours.

Cell absorbance was measured at 450 nm using a microplate reader (Infinite 200 PRO, Tecan, Männedorf, Switzerland). Relative cell viability (%) was determined as follows: Relative viability (%) = ($OD_{experiment} - OD_{blank}$)/($OD_{control} - OD_{blank}$) × 100%, where $OD_{experiment}$, $OD_{control}$, and OD_{blank} represent the OD of the treated cell group, the untreated cell control group, and the cell-free culture medium control group, respectively. For group comparisons, values were normalized to the Control or NC+shNC group.

Flow Cytometric Apoptosis Assay

PC cell apoptosis was assessed using Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (E606336, Sangon Biotech, Shanghai, China) after *PTBP1* silencing and/or *HOXA9* overexpression. In brief, PBS-washed cells were resuspended in 1× binding buffer at 2 × 10^5 cells/mL and incubated with 5 µL Annexin V-FITC for 5 minutes at room temperature in the dark, followed by counterstaining with 5 µL propidium iodide. Finally, the proportion of apoptotic cells was assessed using a flow cytometer (FC500, Beckman-Coulter, Brea, CA, USA).

Assessing Migratory and Invading Capabilities of Transfected Cells

To assess migratory capability, transfected PC cells were resuspended in 100 µL serum-free medium and seeded into the upper chambers of 24-well Transwell inserts (3422, Corning Inc., Corning, NY, USA). The lower chamber was filled with 600 µL medium containing FBS, followed by incubation at 37 °C for 48 hours. Then, the cells were fixed with 4% paraformaldehyde (60536ES60, Yeasen, Shanghai, China) and stained with 0.1% crystal violet (B1087, Applygen Technologies, Beijing, China) at room temperature. Stained cells were counted in four random fields at ×250 magnification using a light microscope (Primovert, ZEISS, Lena, Germany) and analyzed using ImageJ software v2.0.0 (NIH, Bethesda, MD, USA). Migration rate was determined as follows: Relative migration rate (%) = (Cell count in experimental group/Cell count in control group) × 100%. For various group comparisons, values were normalized to the Control or NC+shNC group.

For evaluation of invading capability, 24-well Transwell inserts (3422, Corning Inc., Corning, NY, USA) were pre-coated with Matrigel (354248, Corning Inc., Corning, NY, USA). Following the matrix gel solidification, the same procedure was followed as for the migration assay. Data were analyzed using ImageJ software v2.0.0 (NIH, Bethesda, MD, USA). Invasion rate was assessed as follows: Relative invasion rate (%) = (invasive cell count in the experimental group/invasive cell count in the control group) × 100%. Normalization to the Control or NC+shNC group was performed for comparisons.

Western Blot Analysis

Total protein was isolated from transfected PC cells using RIPA buffer (C500005-0010, Sangon Biotech, Shanghai, China). The cell lysates were centrifuged at 12,000 rpm, and proteins were quantified using the bicinchoninic acid (BCA) Protein Assay Kit (23227, Thermo Fisher, Waltham, MA, USA). An equal amount of proteins (40 µg) was resolved with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes. Membranes were blocked with 5% non-fat milk (R21306, Yuanye Bio-Technology Co., Ltd., Shanghai, China) in tris-buffered saline Tween 20 (TBST; R21284, Yuanye Bio-Technology Co., Ltd., Shanghai, China) at room temperature, and underwent overnight incubation with primary antibodies at 4 °C. The next day, membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for two hours. The blots were then developed using an enhanced chemiluminescent substrate (36222ES60, Yeasen, Shanghai, China) and photographed on a GelDox XR system (Bio-Rad, Hercules, CA, USA). Protein bands were quantified, and relative protein expressions were determined as the gray value of the target protein/internal reference protein. For comparisons, protein expression was normalized to the mean value of the control or NC+shNC group.

Primary antibodies (Abcam, Cambridge, UK) used were as follows: E-cadherin (ab231303, 97 kDa, 1:1000), N-cadherin (ab245117, 100 kDa, 1:1000), Vimentin (ab20346, 54 kDa, 1:1000), Snail (ab216347, 29 kDa, 1:1000), GAPDH (ab181602, 36 kDa, 1:10,000), HOXA9 (A19257, 30 kDa, 1:1000; ABclonal; Wuhan, China). Secondary antibodies (ABclonal; Wuhan, China) applied were goat anti-rabbit IgG (AS014, 1:10,000) and goat anti-mouse IgG (AS003, 1:10,000).

Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM) from at least three independent experiments. The independent *t*-test was employed for comparisons between groups in Fig. 5B,C. Multi-group comparisons were conducted using the one-way analysis of variance followed by Tukey's post hoc test. Statistical analyses were performed in GraphPad Prism v8.0 (GraphPad Software Inc., San Diego, CA, USA), with a *p*-value of less than 0.05 considered statistically significant.

Results

Expression Levels of PTBP1 in PC Cells

GEPIA analysis indicated upregulation of *PTBP1* in PAAD tumor samples (Fig. 1A, $p < 0.05$). Consistently, qRT-PCR verified substantially higher *PTBP1* mRNA levels in PC cell lines (PANC-1 and SW1990) than HPDE6-C7 cells (Fig. 1B, $p < 0.001$).

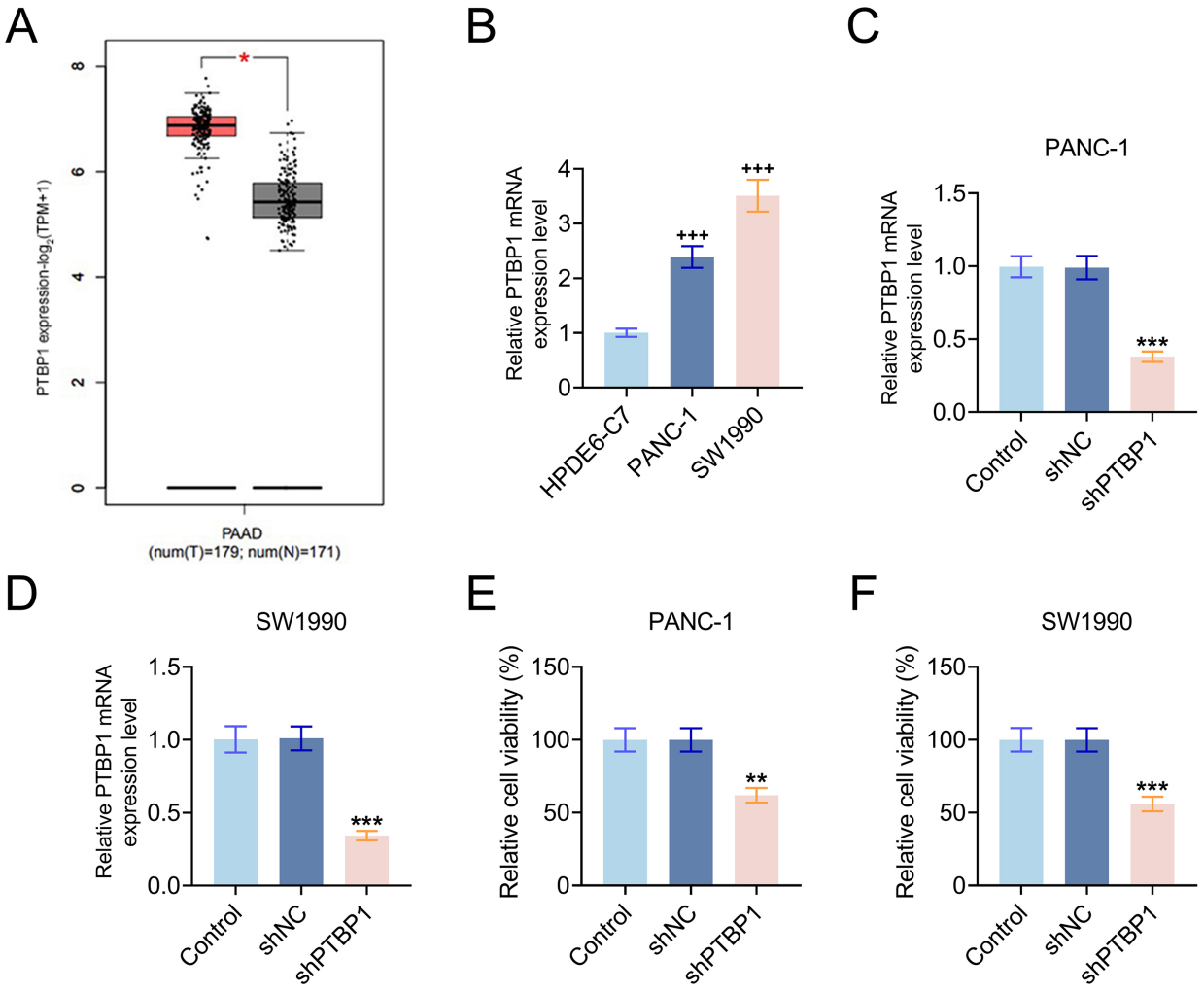

Fig. 1. Expression levels of *PTBP1* in PC cells and its impact on cell viability. (A) Bioinformatics analysis of *PTBP1* expression in 179 tumor samples and 171 normal samples from the GEPIA. (B) *PTBP1* mRNA expressions in human pancreatic duct epithelial cell line (HPDE6-C7) and PC cell lines (PANC-1 and SW1990) (qRT-PCR). (C,D) *PTBP1* mRNA expressions in PC cells after transfection with shPTBP1 or shNC (qRT-PCR), with normal cells as the control (shPTBP1, shNC, and Control groups). (E,F) Cell viability in each group (cell counting kit-8 assay). Data are expressed as mean ± standard deviation (n = 3 independent). *GAPDH* was used as an internal control. $^{*}p < 0.05$, vs. normal; $^{+++}p < 0.001$, vs. HPDE6-C7; $^{**}p < 0.01$, $^{***}p < 0.001$, vs. shNC. Abbreviations: GEPIA, Gene Expression Profiling Interactive Analysis; PTBP1, polypyrimidine tract binding protein 1; PC, pancreatic cancer; PAAD, pancreatic adenocarcinoma; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; shPTBP1, short hairpin RNA (shRNA) targeting *PTBP1*; shNC, shRNA negative control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

PTBP1 Silencing Reduces Viability and Promotes Apoptosis in PC Cells

To explore the function of PTBP1 in PC cells, we performed *in vitro* silencing of *PTBP1*. As illustrated in Fig. 1C,D, transfection with shPTBP1 significantly decreased *PTBP1* expression in PC cells ($p < 0.001$). Compared with shNC control cells, shPTBP1 substantially reduced cell viability (Fig. 1E,F, $p < 0.01$) and significantly increased apoptosis in PC cells (Fig. 2A–C, $p < 0.001$).

PTBP1 Knockdown Reduces Migration, Invasion, and EMT in PC Cells

Transwell assays revealed that shPTBP1 transfection substantially reduced PC cell migration and invasion (Fig. 3A–E, $p < 0.05$). Western blot analysis further showed EMT-related marker shifts consistent with a mesenchymal-to-epithelial transition. Compared to shNC controls, E-cadherin was found to be upregulated, whereas N-cadherin, Vimentin, and Snail were downregulated in shPTBP1-transfected cells (Fig. 4A–M, $p < 0.01$). These observations indicate that *PTBP1* silencing represses EMT in PC cells.

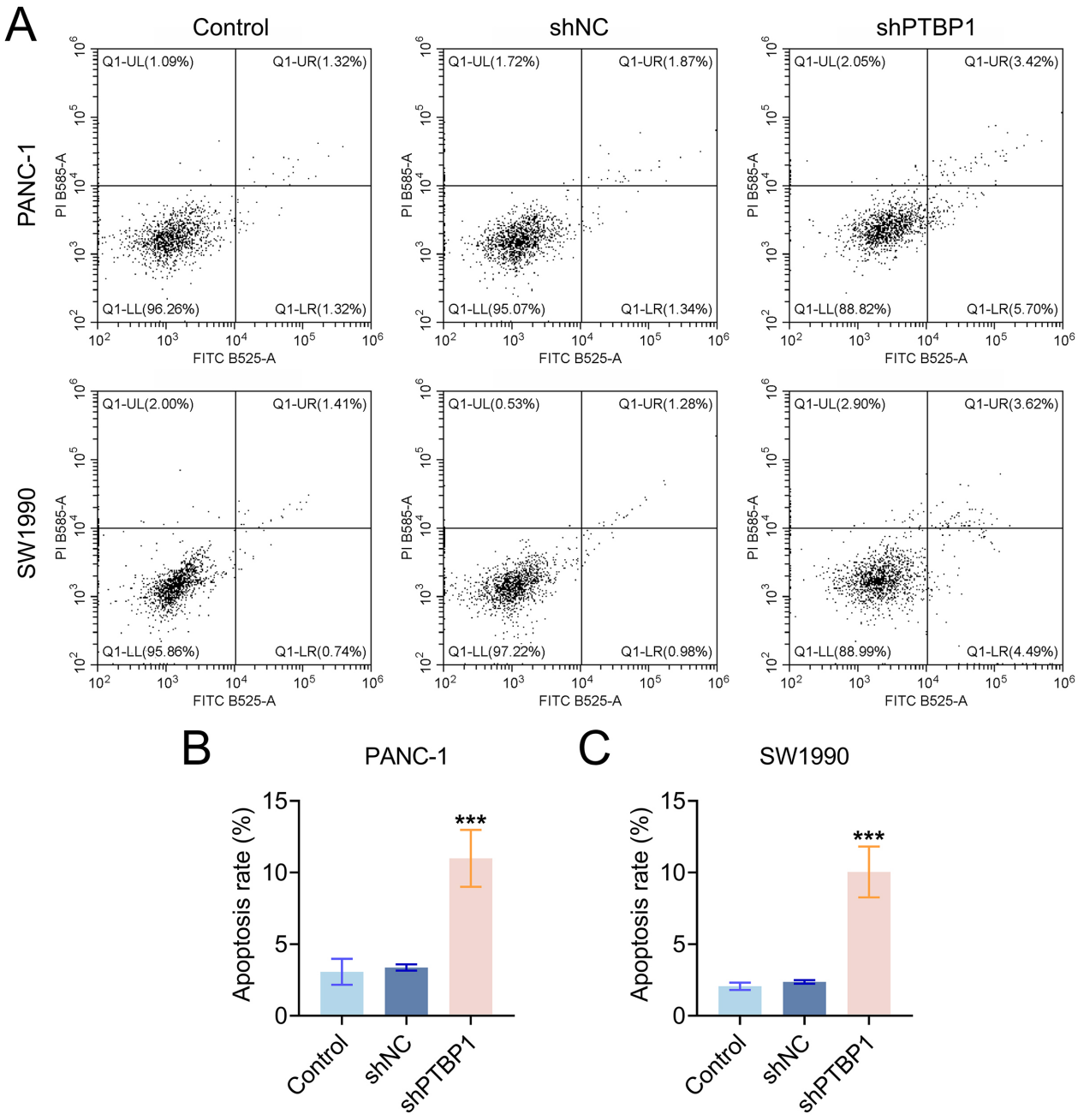

Fig. 2. The effect of PTBP1 on PC cell apoptosis. (A) PC cells apoptosis after transfection with shPTBP1 or shNC, with normal cells as the control (shPTBP1, shNC, and Control groups) (flow cytometry). (B,C) Quantification of apoptosis in PANC-1 (B) and SW1990 (C) cells (flow cytometry). Data are expressed as mean ± standard deviation (n = 3 independent). $^{***}p < 0.001$, vs. shNC.

PTBP1-HOXA9 Interaction Positively Regulates the Expression of HOXA9 in PC

ENCORI analysis demonstrated a positive correlation between *HOXA9* and *PTBP1* expressions in PAAD (Fig. 5A, $r = 0.303$, $p = 3.95 \times 10^{-5}$). Then, we performed RIP assay to validate an RNA-protein binding (*HOXA9*-PTBP1 interaction) in PC cells. The results revealed that more *HOXA9* transcripts were enriched in anti-PTBP1 immunoprecipitants compared to the control (*GAPDH*) (Fig. 5B,C, $p < 0.05$). Consistently, *PTBP1* silencing decreased HOXA9 expression, aligning with findings from Western blot analysis (Fig. 5D,E, $p < 0.001$).

HOXA9 Overexpression Counteracts the Impact of PTBP1 Knockdown in Suppressing the Malignant Behavior of PC Cells

To assess whether *HOXA9* functions downstream of PTBP1, we transfected *HOXA9*-expressing plasmids into

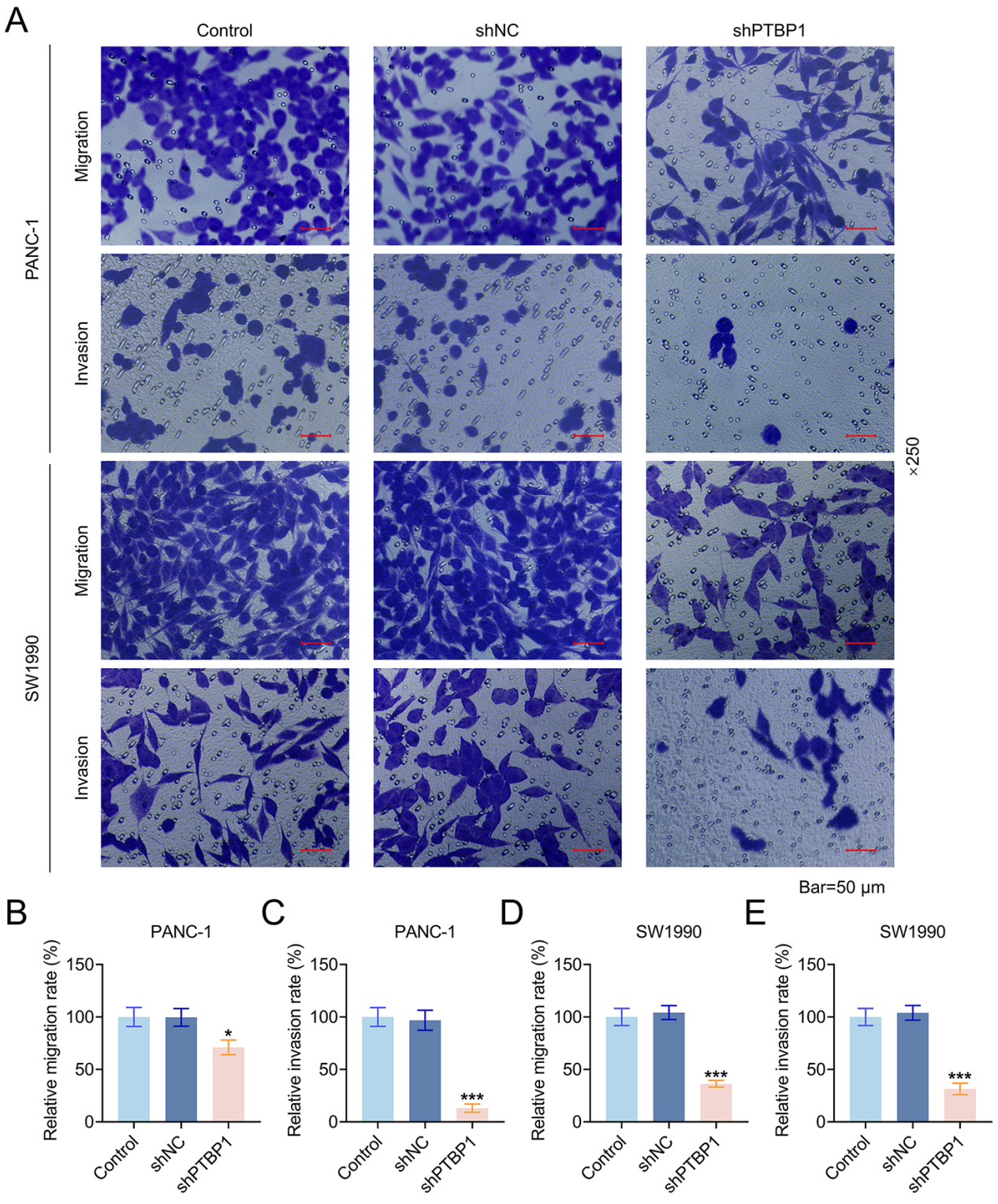

Fig. 3. The effects of *PTBP1* knockdown on the migration and invasion of PC cells. (A) PC cells were transfected with shPTBP1 or shNC, with normal cells as the control (shPTBP1, shNC, and Control groups). Representative images (magnification: ×250, scale bar = 50 μm) of migrated and invaded cells in each group (Transwell assays). (B,D) The quantitative results of the migration in PANC-1 (B) and SW1990 (D) cells. (C,E) Quantification of invasion in PANC-1 (C) and SW1990 (E) cells. Data are expressed as mean ± standard deviation (n = 3 independent). $^{*}p < 0.05$, $^{***}p < 0.001$, vs. shNC.

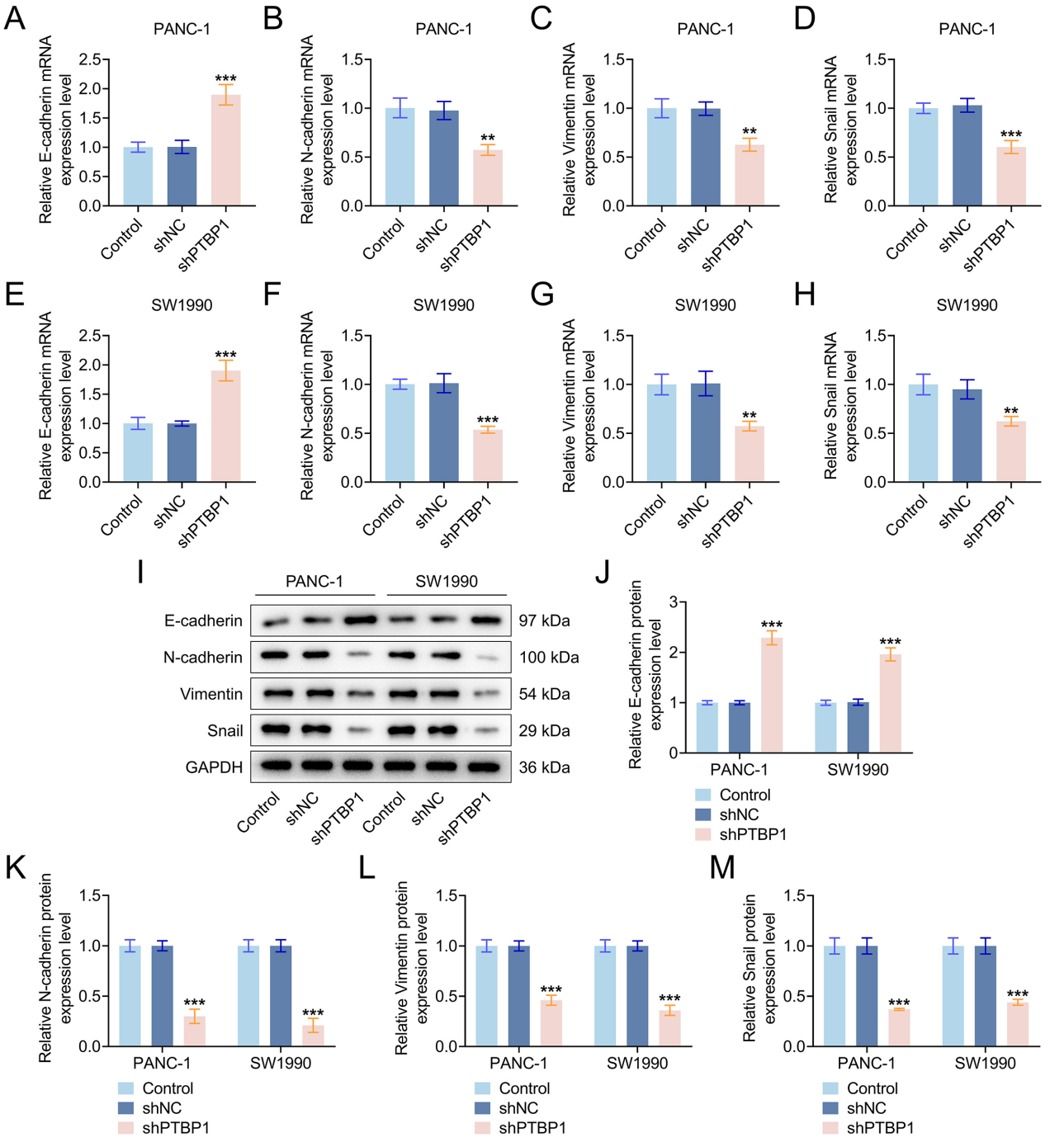

Fig. 4. The effects of *PTBP1* knockdown on EMT-related markers in PC cells. (A–M) PC cells were transfected with shPTBP1 or shNC, with normal cells as the control (shPTBP1, shNC, and Control groups). Expression levels of epithelial-mesenchymal transition (EMT)-related proteins (epithelial cadherin (E-cadherin), neural cadherin (N-cadherin), Vimentin, and Snail) in PANC-1 and SW1990 cells was detected by qRT-PCR ((A–H), PANC-1 (A–D); SW1990 (E–H)) and Western blot analysis (I–M). Data are described as mean ± standard deviation (n = 3 independent). GAPDH served as an endogenous control. $^{**}p < 0.01$, $^{***}p < 0.001$, vs. shNC.

PC cells. qRT-PCR confirmed significant *HOXA9* overexpression (Fig. 6A, $p < 0.001$), and Western blot analysis revealed increased HOXA9 levels in the HOXA9+shNC group than the NC+shNC group (Fig. 6B–D, $p < 0.001$). Notably, the increased expression of *HOXA9* by *HOXA9*-expressing plasmids was reversed in the cells following the transfection of shPTBP1, whereas the suppressing role of shPTBP1 in HOXA9 expression was counteracted in the cells following the transfection of *HOXA9*-expressing plasmids (Fig. 6B–D, $p < 0.001$).

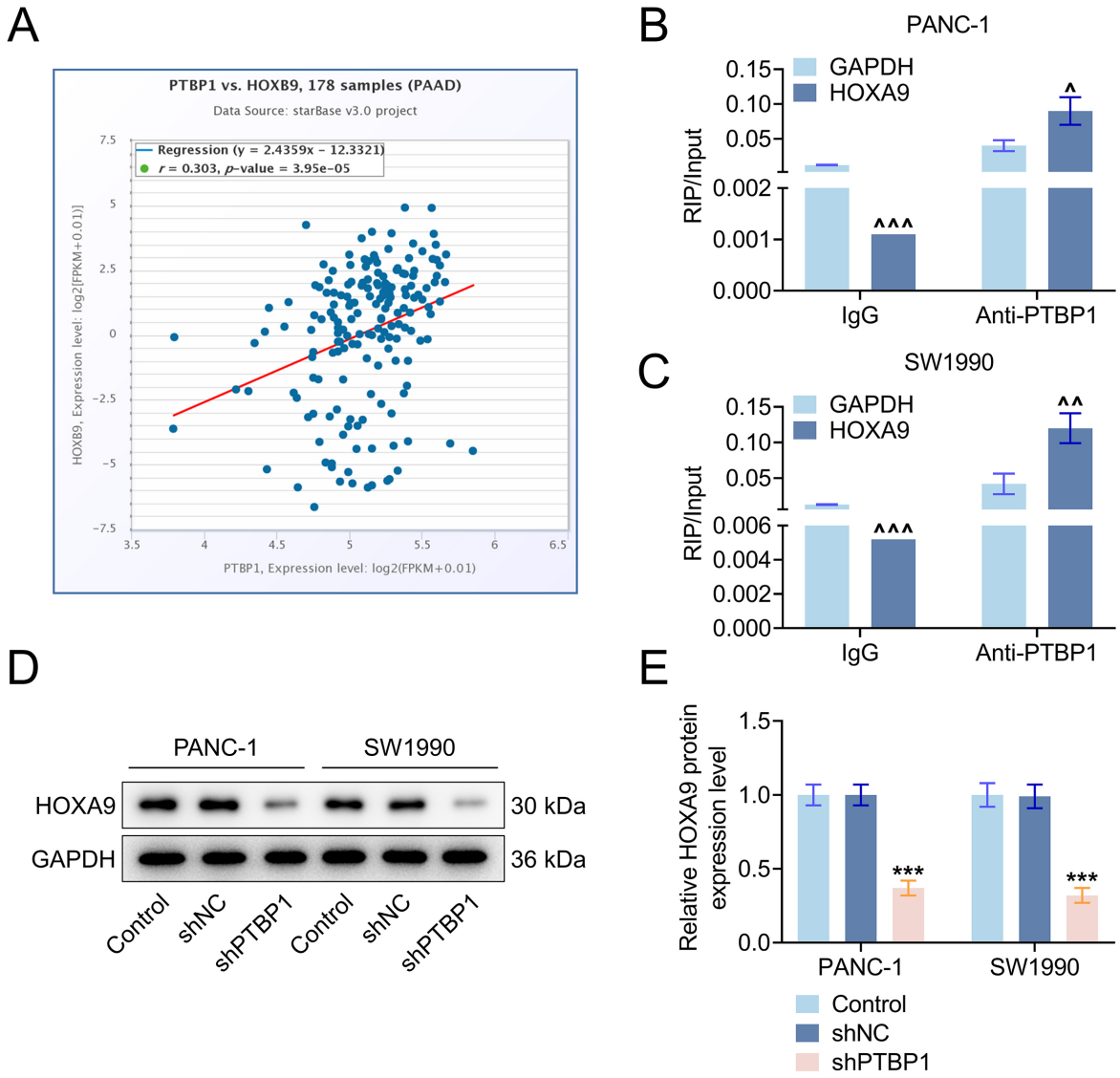

Fig. 5. Interaction of PTBP1 and *HOXA9*, and their correlation in PC cells. (A) Correlation analysis of *PTBP1* and *HOXA9* expressions in 178 PAAD samples. (B,C) Interaction of PTBP1 and *HOXA9* in PC cells (RNA immunoprecipitation assay using anti-PTBP1 antibody). (D,E) PC cells were transfected with shPTBP1 or shNC, with normal cells as the control (shPTBP1, shNC, and Control groups). HOXA9 protein expression in each group (Western blot analysis). Data are expressed as mean ± standard deviation (n = 3 independent). GAPDH served as an endogenous control. $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$, vs. GAPDH; $^{***}p < 0.001$, vs. shNC. Abbreviations: HOXA9, homeobox A9; RIP, RNA immunoprecipitation.

Functionally, *HOXA9* overexpression enhanced viability and reduced apoptosis in PC cells (Fig. 6E–H, $p < 0.05$), effects that were antagonized by *PTBP1* deficiency ($p < 0.001$). Relative to *PTBP1* silencing alone, co-expression of *HOXA9* restored cell viability and further decreased apoptosis (Fig. 6E–H, $p < 0.01$). The Transwell assay demonstrated *HOXA9* overexpression increased PC cell migration and invasion (Fig. 7A–E, $p < 0.001$), which were reversed by *PTBP1* silencing (Fig. 7A–E, $p < 0.01$). Moreover, *HOXA9* overexpression also rescued the migration and invasion deficits caused by *PTBP1* silencing (Fig. 7A–E, $p < 0.05$).

Mechanistically, *HOXA9*-overexpression alleviated E-cadherin and increased N-cadherin, Vimentin, and Snail levels (Fig. 8A–L, $p < 0.001$). On the contrary, *HOXA9* overexpression abrogated the EMT suppression caused by *PTBP1* silencing, restoring the EMT-associated markers (E-cadherin, upregulated N-cadherin, Vimentin, and Snail) profile (Fig. 8A–L, $p < 0.05$).

Discussion

PC is characterized by high invasion and frequent treatment resistance, resulting in extremely low survival

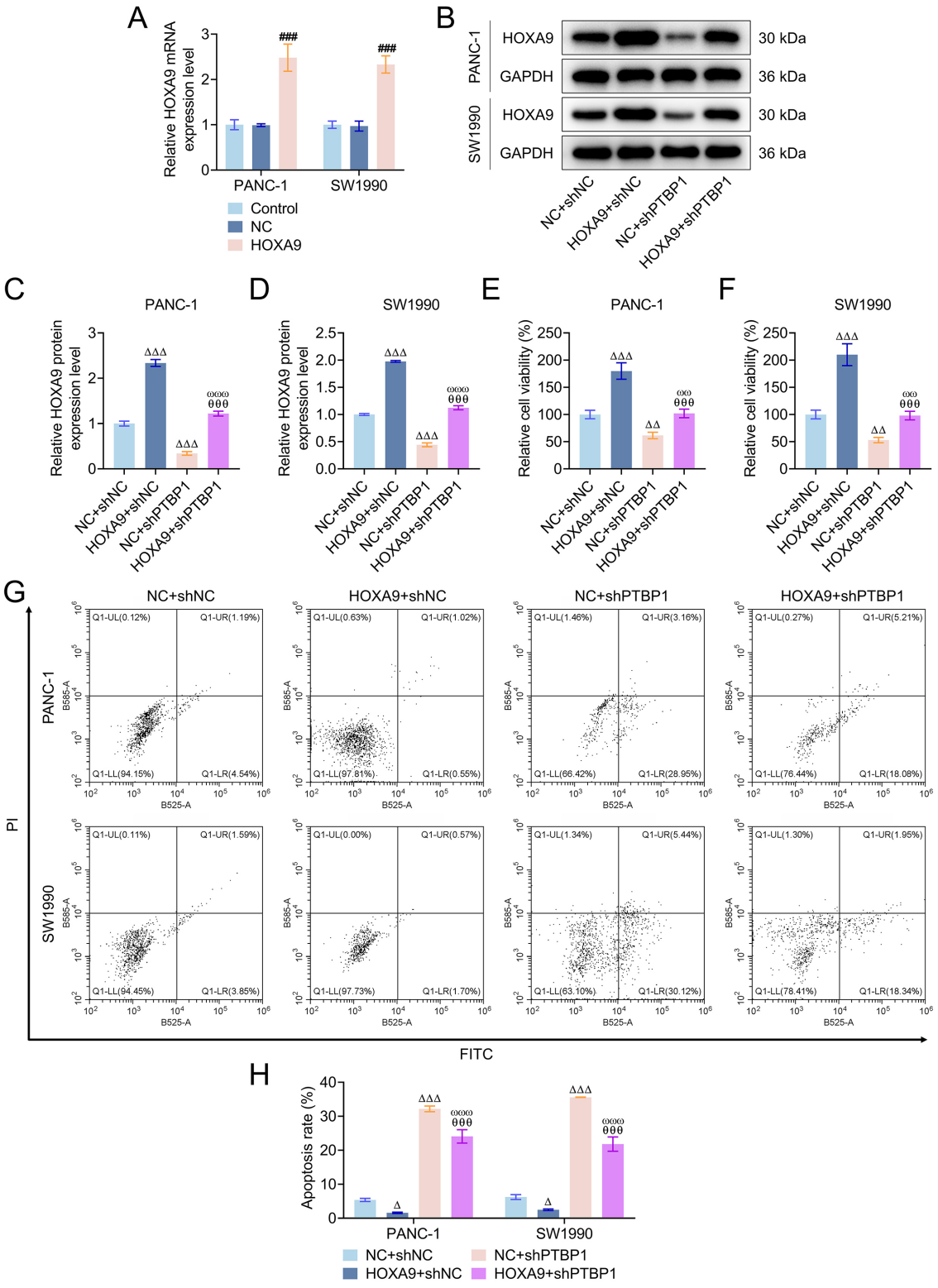

Fig. 6. Role of *HOXA9* in *PTBP1*'s effect on PC cell viability and apoptosis. (A) *HOXA9* mRNA expression in PC cells transfected with *HOXA9*-expressing plasmids or empty vectors, with normal cells as the control (qRT-PCR). (B–D) PC cells transfected with shPTBP1 or/and *HOXA9*-expressing plasmids, with the negative controls-transfected cells as the control (*HOXA9*+shPTBP1, NC+shPTBP1, *HOXA9*+NC and NC+shNC groups). HOXA9 protein expression in each group (Western blot analysis). (E,F) Cell viability (CCK-8 assay). (G,H) Cell apoptosis (flow cytometry). Data are expressed as mean ± standard deviation (n = 3 independent). GAPDH served as an endogenous control. $^{###}p < 0.001$, vs. NC; $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$, vs. NC+shNC; $^{\theta\theta\theta}p < 0.001$, vs. *HOXA9*+shNC; $^{\omega\omega}p < 0.01$, $^{\omega\omega\omega}p < 0.001$, vs. NC+shPTBP1. NC, negative control.

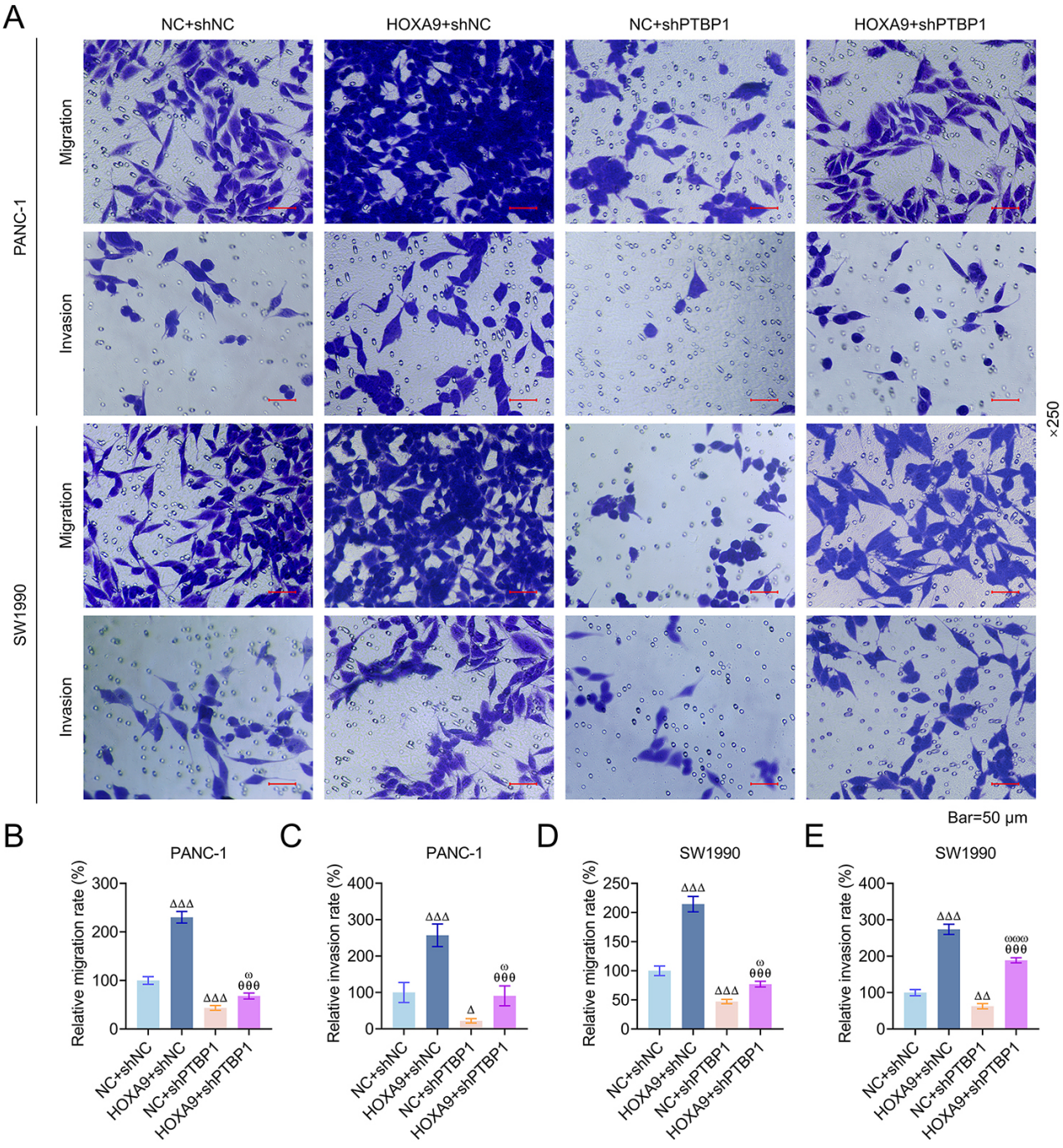

Fig. 7. Role of *HOXA9* in *PTBP1*'s effects on PC cell migration and invasion. (A–E) PC cells transfected with shPTBP1 or/and *HOXA9*-expressing plasmids, with the negative control-transfected cells as the control (*HOXA9*+shPTBP1, NC+shPTBP1, *HOXA9*+NC, and NC+shNC groups). Representative images (magnification: ×250, scale bar = 50 μm) of cell migration and invasion in each group (Transwell assay). Data are expressed as mean ± standard deviation (n = 3 independent). $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$, vs. NC+shNC; $^{\theta\theta\theta}p < 0.001$, vs. *HOXA9*+shNC; $^{\omega}p < 0.05$, $^{\omega\omega\omega}p < 0.001$, vs. NC+shPTBP1.

outcomes [14]. Multiple EMT-associated regulators such as miRNAs and lncRNAs have been implicated in the progression of PC and are considered potential biomarkers and therapeutic targets [15,16]. This study reveals a critical mechanistic role of PTBP1 in PC progression. We demonstrated that PTBP1 is upregulated in PC cells, and its silencing significantly suppresses malignant phenotypes. Mechanistically, PTBP1 drives EMT by upregulating the transcription factor HOXA9; however, *PTBP1* silencing upregulates E-cadherin and downregulates N-cadherin, Vimentin, and Snail. Overexpression of *HOXA9* effectively reversed cellular viability deficits induced by PTBP1 si-

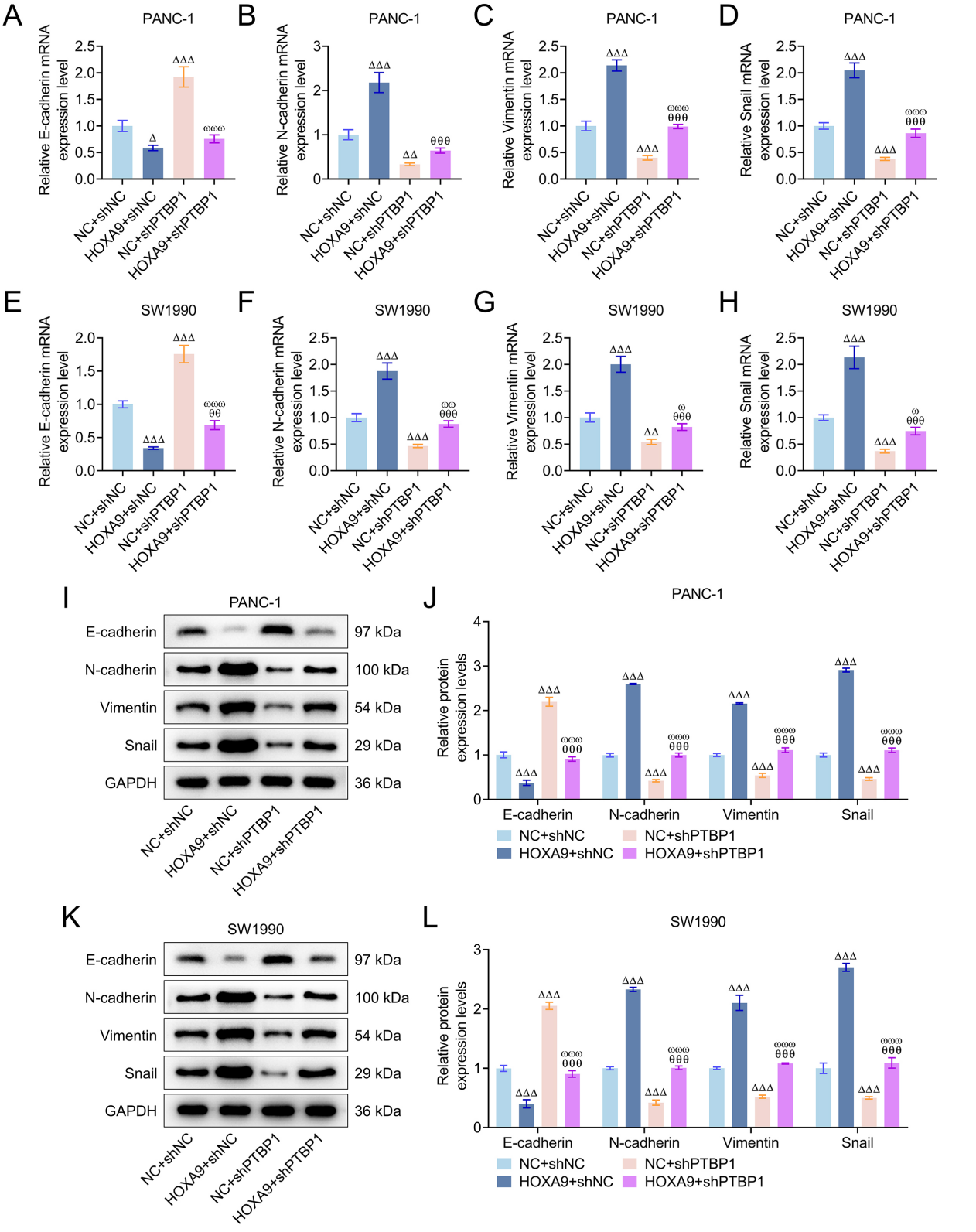

Fig. 8. Role of *HOXA9* in *PTBP1*'s effects on EMT-related markers in PC cells. (A–L) PC cells transfected with shPTBP1 or/and *HOXA9*-expressing plasmids, with the negative control-transfected cells as the control (*HOXA9*+shPTBP1, NC+shPTBP1, *HOXA9*+NC and NC+shNC groups). EMT-related markers (E-cadherin, N-cadherin, Vimentin, and Snail) expressions (qRT-PCR and Western blot analyses). Data are expressed as mean ± standard deviation (n = 3 independent). GAPDH acted as an endogenous control. $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$, vs. NC+shNC; $^{\theta\theta}p < 0.01$, $^{\theta\theta\theta}p < 0.001$, vs. *HOXA9*+shNC; $^{\omega}p < 0.05$, $^{\omega\omega}p < 0.01$, $^{\omega\omega\omega}p < 0.001$, vs. NC+shPTBP1.

lencing, whereas the pro-tumorigenic effects of *HOXA9* overexpression were counteracted by PTBP1 deficiency. Collectively, these findings establish the PTBP1-HOXA9 signaling axis as a key pathway regulating EMT, invasion, and survival in PC cells.

PTBP1 is frequently overexpressed in several types of cancer cells, including PC cells, and promotes tumor progression through multiple pathways [17,18]. In gastric cancer, down-regulation of PTBP1 inhibits cell proliferation [19], and PTBP1 silencing decreases circRNA expression and reduces proliferation [20]. In contrast, *PTBP1* knockdown has been found to increase the mean tumor volume in hepatocellular carcinoma [21], suggesting its diverse roles across various cancers. In PC, PTBP1 has been implicated through its interaction with heterogeneous nuclear ribonucleoprotein L whose expression is positively correlated with tumor–node–metastasis (TNM) stage and pathological grade [22]. However, the mechanism by which PTBP1 influences the malignant behavior of PC cells is yet to be properly elucidated.

Our results revealed that *PTBP1* knockdown inhibits PC cell viability, migration, and invasion. Given that EMT activation presents subsets of PC cells with stemness characteristics, including self-renewal and improved viability [14,23], and *PTBP1*'s positive role in forming stem-like cells in gastric cancer [13], our study offers the first systematic loss-of-function analysis of *PTBP1* in PC and directly demonstrates its regulatory role in EMT. Furthermore, as Snail is an established determinant of EMT initiation that supports survival and dissemination of cancer cells [24,25], our findings place *PTBP1* upstream of the Snail regulatory network, advancing the mechanistic framework of EMT in PC.

Mechanistically, this study observes that PTBP1 enhances *HOXA9* expression through RNA-protein binding manner. The *HOXA9* gene, a homeobox transcription factor, is an oncogene in many cancers, including head and neck squamous cell carcinoma and breast cancer, where it regulates cell proliferation, EMT, invasion, and drug resistance [26–29]. However, its role in PC remains largely uninvestigated, and its regulatory mechanism is unclear. *HOXAs* are essential for maintaining pluripotency, differentiation, and self-renewal of embryonic and hematopoietic stem cells [30,31]. Previous studies have primarily focused on *HOXA9* regulation by protein-coding genes such as hypoxia-inducible factor-1α (HIF-1α) and TET1 [28,29] or by non-coding RNAs like lncRNA HOTTIP [32]. Consistent with this, our previous study revealed that miR-210 promotes EMT and reduces gemcitabine sensitivity in PC cells under hypoxic conditions by inhibiting *HOXA9* and activating the nuclear factor kappa-B (NF-κB) pathway [33]. In this study, we found that *HOXA9* overexpression reduces apoptosis and strengthens migration, invasion, and EMT in PC cells. Furthermore, *HOXA9* has been highlighted to be partially required for Twist1-mediated EMT; its knockdown alleviates Twist1-induced invasion of prostate cancer cells *in vitro* and limits metastasis *in vivo* [34].

Collectively, these observations support *HOXA9* as a potential biomarker associated with EMT in PC. Furthermore, we observed that the influences of overexpressed *HOXA9* and *PTBP1* silencing on the malignant behavior of PC cells were mutually reversed, indicating the PTBP1-HOXA9 axis might be a driver of EMT and disease progression in PC. This observation provides a mechanistic basis for PC invasion and metastasis. Moreover, we found that silencing *PTBP1* significantly increased the number of late apoptotic cells, suggesting that *PTBP1* deficiency may trigger an irreversible process in the execution of apoptosis. Notably, *HOXA9* overexpression significantly rescued *PTBP1* silencing-induced late apoptosis, confirming that the PTBP1-HOXA9 axis primarily supports cell survival by suppressing late-stage apoptosis, a hypothesis that requires further experimental validation.

These findings carry critical potential clinical implications. PTBP1 and HOXA9 expressions have been linked to the clinicopathological features, such as stage, grade, and metastasis, in various cancers [35,36]. However, this study did not include a clinical correlation between PTBP1/HOXA9 expression and clinicopathological features. Future studies should validate the association between PTBP1/HOXA9 expression and these characteristics through clinical data, which could provide a basis for risk stratification and guide treatment or intervention in PC.

We acknowledge several limitations in this study. First, PTBP1 expression was not assessed in clinical specimens or animal models, and no *in vivo* experiment (e.g., mouse xenograft models) was conducted to further validate the *in vitro* results; furthermore, Western blot verification for the relevant genes was also not included. The role of the PTBP1/HOXA9 axis in promoting PC progression through EMT needs *in vivo* validation. Second, the downstream mechanism by which *HOXA9* transcriptionally regulates EMT-related genes remains unclear. The impact of *HOXA9* on the EMT gene network is currently unknown, warranting investigations such as chromatin immunoprecipitation sequencing (ChIP-seq) and functional assays to identify direct *HOXA9* targets and relevant signaling pathways. Finally, the current functional evidence primarily relies on genetic manipulations (gene knockdown and overexpression). Future validation using pharmacological tools targeting PTBP1 or HOXA9 (when available) will provide stronger translational relevance of these findings for therapeutic applications.

Conclusions

In summary, our current findings unveil a novel metastatic mechanism of PC and provide compelling evidence that the upregulation of PTBP1 enhances *HOXA9* ex-

pression, thereby promoting EMT and driving the invasiveness of PC cells *in vitro*. These results underscore PTBP1 as a potential molecular target for EMT-directed interventions aimed at inhibiting stemness acquisition and alleviating tumor progression in PC.

Availability of Data and Materials

The datasets generated during and analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

NZ contributed substantially to the conception and design of the study. JN, XWH, JL, and QL were responsible for data acquisition, data analysis, and interpretation. QL drafted the manuscript, and all authors revised it critically for important intellectual content. All authors approved the final version to be published and agreed to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202537200.161.

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