

Effect and Mechanism of LncRNA-PBC11 in Regulating EMT Progression of Prostate Cancer Cells

Haoyu Cui (✉ cuihaoyu6@126.com)

Jinshan Hospital of Fudan University <https://orcid.org/0000-0002-5950-4452>

Xuefeng Xie

Jinshan Hospital of Fudan University <https://orcid.org/0000-0002-7436-8006>

Chuansheng Hou

Jinshan Hospital of Fudan University

Qiang Ma

Jinshan Hospital of Fudan University

Zhuo Chen

Jinshan Hospital of Fudan University

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Abstract

The invasion and metastasis of prostate cancer is the main reason for its poor prognosis. Up to now, ADT (androgen deprivation therapy, ADT), whether surgical or drug therapy, is the basic method for the treatment of prostate cancer. However, ADT therapy is generally not recommended for the clinical treatment of metastatic prostate cancer. EMT (epithelial-to-mesenchymal transition, EMT) of tumor cells is the internal driving factor of tumor invasion and metastasis. Therefore, in-depth study on the regulation mechanism of EMT in prostate cancer cells will help us develop the new prevention and treatment plan for prostate cancer. Here, we will demonstrate and elaborate an lncRNA-PBC11 closely related to EMT of prostate cancer cells. The research data show that the content of PBC11 in metastatic prostate tumors increases significantly compared to non-metastatic, and can competitively bind hsa-miR-137 with 3'UTR of Twist1 in the form of miRNA sponge, thereby weakening the antitumor activity of hsa-miR-137. Our functional experiments confirmed that PBC11 knockdown is an important means for the normal operation of EMT inhibitory pathway hsa-miR-137/Twist1 in prostate cancer cells, and PBC11 knockdown combined with hsa-miR-137 overexpression showed excellent resistance to EMT in prostate cancer cells induced by TGF- β 1. In a word, our study shows that PBC11 up-regulates the expression of Twist1 protein by blocking hsa-miR-137/ Twist1 pathway and promotes the progress of EMT in prostate cancer cells, overexpression of PBC11 can effectively inhibit EMT in prostate cancer cells by restoring the negative regulation of Twist1 by hsa-miR-137.

Introduction

In recent years, prostate cancer has the second highest incidence of the global male population rate in all tumor types. Clinical statistics show that the incidence rate of prostate cancer is increasing rapidly among men over 50 years old, and the mortality rate is significantly higher in patients over 85 years old. Thanks to the improvement of early screening and the development of treatment methods, the overall survival rate of prostate cancer patients has been significantly improved [1–3]. Even so, the treatment of MPC (metastatic prostate cancer, MPC) is a major challenge facing the medical community, which is more severe due to the aging of the population and the improvement of the diagnosis rate. At present, the treatment of prostate cancer is mainly through inhibiting androgen receptor activity. The standard treatment for hormone sensitive prostate cancer is ADT (androgen deprivation therapy, ADT) [4–5]. Although more than 80% of prostate cancer patients respond to ADT, a large number of patients are prone to side effects. Although a variety of new therapies and drugs have been used for the treatment of advanced and metastatic prostate cancer, but it will develop into CRPC (castration resistant prostate cancer, CRPC) after an average of 24 months [6]. Serum PSA can be used as a response index for prostate cancer treatment, but it cannot predict the development of CRPC according to the serum PSA value [7]. Therefore, the identification of new potential biomarkers and therapeutic targets is very important to improve and replace the existing clinical therapies for prostate cancer. Here, we report for the first time that the lncRNA-PBC11 has a positive correlation with prostate cancer metastasis. The mechanism study shows that PBC11 can inhibit the tumor suppressor pathway hsa-miR-137/Twist1 in

the form of miRNA sponge, and its knockdown can effectively inhibit EMT of prostate cancer cells induced by TGF- β 1 by restoring the function of hsa-miR-137. Hsa-miR137 overexpression combined with PBC11 knockdown has more advantages than PBC11 knockdown in combating EMT progression of prostate cancer cells. We believe that this study will not only further enrich the molecular mechanism of prostate cancer malignancy, but also provide a strong theoretical support for hsa-miR137 and PBC11 as the new biological targets for prostate cancer patients.

Materials And Methods

Cell culture

Prostate cancer cells LNCaP and PC3 purchased from National Collection of Authenticated Cell Cultures (Shanghai, China) were maintained in F-12K medium (Thermo Fisher, CA, USA) supplemented with 10% fetal calf serum (FBS, Thermo Fisher). The lentiviral packaging cell line, 293T purchased from American Type Culture Collection (VA, USA) were maintained in Dulbecco's modified Eagle's DMEM medium (Dulbecco's modified Eagle's medium, DMEM, Thermo Fisher) supplemented with 10% FBS. All adherent cells were passaged by digestion with 0.25% trypsin (Thermo Fisher) once the cells reached a density of 70%, and were cultured in an atmosphere with saturated humidity at 37°C containing 5% CO₂.

Tumor tissues

24 pairs of metastatic and non-metastatic prostate tumors with their precancerous lesions were collected from 24 prostate patients (12 cases of each group) in the Department of Jinshan Hospital of Fudan University. There was no age limit for enrollment. Informed consent was obtained from all patients in accordance with the standards set by the Ethics Committee of the Jinshan Hospital of Fudan University. Clinicopathological information pertaining to the patients and tumor classification is provided in **Table 1**. Total RNA extracted from the tissues was used for the detection of PBC11, hsa-miR-137 and Twist1 mRNA levels by using RT-qPCR (realtime quantitative PCR). Total protein was extracted from the tissues and used to detect the proteins of Twist1, E-cadherin and Vimentin by western blotting. The detection indexes were used for correlation analysis and evaluation of EMT of tumor cells.

Lentivirus mediated hsa-miR-137 overexpression and PBC11 silencing in prostate cancer cells

Human genomic DNA was extracted and used for amplification of the precursor of has-miR-137(270bp) with the primers 5'-TGCTACCTTGGCAACCACGGGCG-3' and 5'-GGGCGGGCTCAGCGAGCAGCAA-3'. The PCR product was digested and ligated to expression vector to construct pCDH-miR-137. An siRNA (5'-GCTACTCTCACAGCAGCAC-3') sequence complementary to PBC11 was chosen, and the corresponding shRNA oligonucleotide DNA synthesized were annealed and cloned to construct pSIH1-shRNA-PBC11. An siRNA with a scrambled sequence (5'-ACGACCCGCTTAGCCATAC-3') was used as a NC (negative control, NC) to construct pSIH1-NC. The recombinant vectors were confirmed by DNA sequencing and endotoxin free DNA was prepared in all cases.

A total of 1×10^6 293T cells in logarithmic growth phase were plated in 10-cm dishes in 10 ml DMEM supplemented with 10% FBS and cultured overnight under normal conditions. Recombinant vectors (2 μ g each of pCDH-miR-137, pSIH1-shRNA-PBC11 or pSIH1-NC) and 10 μ g of the pPACK packaging plasmids (System Biosciences, CA, USA) were co-transfected into 293T using Lipofectamine 2000 transfection reagent (Thermo Fisher). The culture medium was completely replaced with DMEM containing 1% FBS prior to transfection. After 48 h of transfection, the supernatant was harvested and cleared by centrifugation at 5,000 \times g for 10 min at 4°C and then passed through a 0.45 μ m polyvinylidene difluoride membrane (Millipore, MI, USA). The viral titer was determined using a gradient dilution method. The recombinant lentiviruses were named Lv-miR-137, Lv-shRNA-PBC11 and Lv-NC, respectively.

LNCaP and PC3 cells in logarithmic phase were plated in 6-well plates at a density of 2×10^5 cells/well. One day later, lentiviruses (Lv-NC, Lv-miR-137 and Lv-PBC11) were added to the cells at an MOI (multiplicity of infection, MOI) of 10. The infection efficiency was evaluated by observing the fluorescence of GFP (green fluorescent protein, GFP) 72 hour after infection. Total RNA were isolated and subjected to RT-qPCR to analyze the levels of hsa-miR-137 and PBC11.

Verification of the binding sites of hsa-miR137 in Twist1-3' UTR (untranslated region, UTR)

We used Targetscan7.1 (Whitehead Institute for Biomedical Research) to predict the potential hsa-miR-137 binding sites in the 3'-UTR of human Twist1 mRNA (NM_000474.4). Primers 5' - TTGAGGACCCATGGTAAATGC-3' (forward) and 5'-TTTATATTTATTTATTGCAGAA-3' (reverse) targeting the 3'-UTR of the Twist1 gene were designed and used to introduce the 109 bp PCR product containing the hsa-miR-137 target site (5'-GCAATAA-3'). The PCR product was digested and cloned into pGL3-promoter luciferase reporter vector (Promega Corporation, Madison, WI, USA) to generate the vector pGL3-wt (wild-type)-Twist1. The binding sites in the pGL3-wt-Twist1 was mutated from 5'- GCAATAA -3' to 5'- AAAGATC -3' to construct the mutated reporter vector pGL3-mt-Twist1. The hsa-miR-137 mimics (5'- GAUGC GCAUAAGAAUUCGUUAUUt-3'), inhibitor (5'-AAUAACGAAUUCUUAUGCGCAUCt-3'), and NC (5'- CGUUA AUGCAAUAGGAUGCAUUt-3') were all chemically synthesized by Sangon Biotech (Shanghai, China). 293T cells were co-transfected with the hsa-miR-137 mimics, inhibitor, NC, and pGL-wt-Twist1 or pGL3-mt-Twist1 using Lipofectamine2000 according to the manufacturer's instructions. The cells were harvested, and luciferase assays were performed using a Dual Luciferase Reporter Assay System (Promega) 48 hours after transfection. The effect of PBC11 depletion on the inhibition of luciferase activity by the hsa-miR-137 mimics was evaluated in 293T cells by co-transfection of hsa-miR-137 mimics, pGL3-wt-Twist1 and pcDH-PBC11.

Cell proliferative, invasion and migration assay

LNCaP and PC3 cells were used for cell viability assay by using a cell counting kit-8 assay kit (CCK-8, Dojindo, Japan). LNCaP cells were used for invasion assay by using the QCMTM 24-well Fluorimetric Cell Invasion Assay kit (Millipore) according to the manufacturer's instructions. Migration ability of PC3 cells was assessed using wound healing assay. The cells used in the experiments were infected with the

lentiviruses (Lv-NC or Lv-miR-137 or Lv-shRNA-PBC11 or Lv-miR-137+ Lv-shRNA-PBC11) for 72 hours, then EMT model was established by adding TGF- β 1 (5 ng/ml) for 48 hours.

RIP (RNA Binding Protein Immunoprecipitation, RIP)

We identified the binding of Twist1 to PBC11 in LNCaP cells by RIP experiment. Twist1 protein in cells was precipitated by IP and its bound RNA was obtained from eluent. Then, the target product (PBC11) in the purified product was qualitatively detected by RT-PCR. The specific scheme of RIP refers to Ahmad m Khalil's research [8]. The 2 μ g Twist1 primary antibody (Abcam, Cambridge, UK) was used for IP, and the PCR primer sequence used for target product identification is 5'-AACCACGTGCCCCACAGCGG-3' (forward) and 5'-CTCTGGTGGATGCTGGTTATT-3' (reverse).

RT-qPCR

Total RNA was isolated and reverse-transcribed into cDNA using M-MLV reverse transcriptase. RT-qPCR was performed using the SYBR Premix Ex Taq™ kit and TP800 System (Takara, Dalian, China). The PCRs were carried out under the following conditions: 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and extension at 72°C for 20 s, using cDNA (200 ng) as the template. The levels of Twist1 were normalized to the expression of the endogenous housekeeping gene, β -actin, using the $2^{-\Delta Ct}$ method. The PCR primers used were: Twist1, 5'-TCATGGCCAACGTGCGGGAG-3' (forward) and 5'-TTGTCCGAGGGCAGCGTGGGGATGA-3' (reverse) and β -actin, 5'-CCTGTACGCCAACACAGTGC-3' (forward) and 5'-ATACTCCTGCTTGCTGATCC-3' (reverse). To normalize the level of hsa-miR-137, the level of U6 snRNA was used as reference. The specific primers used for reverse transcription were random9 for the U6 snRNA and 5'-GTCGTATCCAGTGC GTGTCGTGGAGTCGGCAATTGCACTGGATACGAAATAAC-3' for hsa-miR-137. The PCR primers were as follows: U6 snRNA, 5'-GTGCTCGCTTCGGCAGCACAT-3' (forward) and 5'-TACCTTGCGAAGTGCTTAAAC-3' (reverse); hsa-miR-137, 5'-GCCGGCGCCCGAGCTCTGGCTC-3' (forward) and 5'-GATGCGCATAAGAATTCGTTATT-3' (reverse).

Western blotting

Total protein was extracted from cells using the M-PER mammalian protein extraction reagent or from tissues using the T-PER tissue protein extraction reagent (Thermo Fisher). Equal amounts of total proteins (12 μ g) were separated by SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis, SDS-PAGE, 11% gel) and transferred onto nitrocellulose membranes (Millipore). The blots were probed with primary antibodies against human Twist1 (1:500), E-cadherin (1:500), Vimentin (1:600), N-cadherin (1:500), AKT1 (1:400) and β -actin (1:1000) (Abcam), followed by probing with the corresponding secondary HRP-conjugated anti-rabbit antibody (Abcam). After washing the membranes, the bands were detected by chemiluminescence and imaged with X-ray films. β -actin was used as an endogenous reference for normalization.

Statistical analysis

The data are shown as the means \pm standard deviation (SD) of three independent experiments. All statistical data were analyzed using SPSS GradPack version 20.0 statistical software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons between groups were performed using a two-tailed Student's *t*-test or one-way ANOVA with a post-hoc Tukey test. Differences were considered statistically significant when $p < 0.05$.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Results

Twist1 protein is highly expressed in metastatic prostate tumors which is attributed to abnormal posttranscriptional processing

RT-qPCR data showed that the mRNA level of Twist1 was higher in tumors than that in its precancerous lesions ($P < 0.05$ vs. precancerous lesions), but there was no significant difference between metastatic and non-metastatic tumors ($P > 0.05$ vs. non-metastatic tumors. **Fig. 1A**). Analysis of western blotting showed that Twist1 was higher in tumors than that in its precancerous lesions ($P < 0.05$ vs. precancerous lesions), and but there was a significant difference between metastatic and non-metastatic tumors ($P < 0.01$, vs. non-metastatic tumors. **Fig. 1B**). Bioinformatics analysis identified a seven-base hsa-miR-137 seed sequence 5'-GCAAUAA-3' in the 3'-UTR of Twist1 mRNA (**Fig. 1C**). Luciferase experimental data showed that both pGL3-wt-twist1 and pGL3-mt-twist1 could significantly up regulate the luciferase activity in 293T cells 48 hours after transfection ($P < 0.01$, vs 293T cells). Hsa-miR-137 overexpression and inhibition (via transfection of hsa-miR-137 mimics or inhibitor) can only inhibit the increase of luciferase in 293T cells caused by pGL3-wt-twist1 ($P < 0.01$, vs 293T cells transfected with pGL3-wt-Twist1), but have no significant effect on the increase of luciferase in 293 cells caused by pGL3-mt-Twist1 ($P > 0.05$, vs 293T cells transfected with pGL3-mt-Twist1). Co-transfection of hsa-miR-137 NC had no effect on the luciferase activity of either of the two vectors ($p > 0.05$, vs. 293T cells transfected with pGL3-wt-Twist1 or pGL3-mt-Twist1) (**Fig. 1D**). RT-qPCR data also showed that the level of hsa-miR-137 was lower in tumors than that in its precancerous lesions ($P < 0.05$ vs. precancerous lesions), but there was no significant difference between metastatic and non-metastatic tumors ($P > 0.05$ vs. non-metastatic tumors. **Fig. 1E**). Comprehensive analysis data revealed that in the non-metastatic tumors, the hsa-miR-137 expression was negatively correlated with that of Twist1 protein. However, in metastatic tumors, there was no expression correlation between them (**Fig. 1F**).

Hsa-miR-137 loses its negative regulation on Twist1 is related to the high expression of PBC11 in metastatic prostate tumors

RT-qPCR data showed that the level of PBC11 was the highest in metastatic prostate tumors ($P < 0.01$ vs. the other three groups), and there was no significant difference between non-metastatic prostate tumors

and the two precancerous lesions ($P < 0.05$, **Fig. 2A**). Comprehensive analysis data revealed that in the non-metastatic tumors, the PBC11 expression was positively correlated with Twist1 protein in both metastatic and non-metastatic prostate tumors (**Fig. 2B**). Bioinformatics analysis indicated the existence of six 7-base hsa-miR-137 binding sites in the PBC11 (**Fig. 2C**). The results of the luciferase assay showed that hsa-miR-137 mimics lost their inhibitory effect on luciferase activity caused by of the pGL3-wt-Twist1 in 293T cells following the transfection of pcDH-PBC11 (**Fig. 2D**). RIP was used for analyze the direct binding of Twist1 to PBC11, and a negative result was obtained (**Fig. 2E**).

Lentivirus mediated hsa-miR137 overexpression and Twist1 gene silencing in prostate cancer cell lines LNCaP and PC3

After being infected with lentiviruses for 72 hours, the results of cell counting showed that the majority of cells in the same field of vision have the GFP expression, indicating that the lentivirus infection efficiency of the two cells was close to 100% (**Fig. 3AB**, upper). Data of RT-qPCR showed that hsa-miR-137 level significantly increased in the Lv-miR-137 group ($p < 0.01$ vs. cells or Lv-NC group or Lv-shRNA-PBC11 group), and there was no significant difference between the cells, Lv-NC and Lv-shRNA-PBC11 groups ($P > 0.05$). Data of RT-qPCR also showed that PBC11 level significantly decreased in the Lv-shRNA-PBC11 group ($p < 0.01$ vs. cells or Lv-NC group or Lv-miR-137 group), and there was no significant difference between the cells, Lv-NC and Lv-miR-137 groups ($P > 0.05$) (**Fig. 3A B**, down).

PBC11 knockdown can ensure the effect of hsa-miR-137 on the inhibiting of EMT in prostate cancer cells induced by TGF- β 1

Cell proliferation assay data showed that induction of TGF- β 1 significantly enhanced the proliferation of LNCaP and PC3 cells with a significant difference ($P < 0.05$ vs. cells not treated). The overexpression of hsa-miR-137 slightly suppressed the proliferation of LNCaP and PC3 cells treated with TGF- β 1 but there was no significant difference between them ($P > 0.05$ vs. cells treated with TGF- β 1). The silence of PBC11 suppressed the proliferation of LNCaP and PC3 cells induction by TGF- β 1 with a significant differences ($P < 0.05$ vs. cells treated with TGF- β 1). Compared with overexpression of hsa-miR-137 or silence of PBC11, overexpression of hsa-miR-137 combined silence of PBC11 could significantly inhibit the proliferation activity of tumor cells under TGF- β 1 induction ($P < 0.01$ vs. cells treated with TGF- β 1, **Fig. 4A**). Data of cell invasion assays showed that the invasive ability significantly enhanced in LNCaP cells under TGF- β 1 induction ($P < 0.01$ vs. cells not treated). The overexpression of hsa-miR-137 or silence of PBC11 slightly suppressed the invasion of LNCaP cells treated with TGF- β 1, but there was no significant difference between groups ($P > 0.05$ vs. cells treated with TGF- β 1). Compared with overexpression of hsa-miR-137 or silence of PBC11, overexpression of hsa-miR-137 combined silence of PBC11 could significantly inhibit the invasion of tumor cells under TGF- β 1 induction ($P < 0.01$ vs. cells treated with TGF- β 1, $P < 0.05$ vs. cells treated with TGF- β 1 with infection of Lv-miR-137 or Lv-shRNA-PBC11.) (**Fig. 4B**). The cell migration detection data in PC3 cells showed that the change of cell migration in each treatment groups was completely consistent with the invasion of LNCaP cells (**Fig. 4C**).

The progression of prostate cancer metastasis is related to EMT of tumor cells and PBC11 silencing combined with has-miR-137 overexpression can effectively inhibit TGF- β 1 induced EMT in LNCaP cells

Western blotting result showed that expression of E-cadherin was low in non-metastatic tumors than that in its precancerous lesions but there was no significant difference between them ($P \geq 0.05$ vs. precancerous lesions), was lower in metastatic tumors than that in its precancerous lesions with a significant difference ($P \leq 0.01$ vs. precancerous lesions), and was lower in metastatic tumors than that in non-metastatic tumors with a significant difference ($P \leq 0.01$ vs. non-metastatic tumors). The results of western blot also showed that the expression of Vimentin in each group was completely opposite to that of E-cadherin. **Fig.5A**). RT-qPCR results showed that TGF- β 1 induction for 48 hours had no significant effect on the content of hsa-miR137 in LNCaP cells ($P \geq 0.05$ vs. cells not treated), while Lv-miR-137 or Lv-miR-137 combined Lv-shRNA-PBC11 infection could significantly up-regulate its levels in LNCaP cells induced by TGF- β 1 ($P \leq 0.01$ vs. cells induced by TGF- β 1). Induction of TGF- β 1 for 48 hours significantly increased PBC11 level in LNCaP cells ($P \leq 0.05$ vs. cells not treated), and hsa-miR-137 overexpression had no significant effect on the content of PBC11 in LNCaP cells induced by TGF- β 1 ($P \geq 0.05$ vs. cells induced by TGF- β 1), hsa-miR-137 decreased significantly in Lv-miR-137 combined Lv-shRNA-PBC11 infection and TGF- β 1 induced cells ($P \leq 0.05$ vs. cells induced by TGF- β 1 group or cells induced by TGF- β 1+Lv-miR-137 infection group) (**Fig.5B**). The results of western blotting also showed that compared with the cell group, the expression of Twist1 could be significantly up-regulated by TGF- β 1 induction for 48 hours ($P \leq 0.01$ vs. cells not treated), while Lv-miR-137 or Lv-NC infection had no effect on Twist1 protein in LNCaP cells induced by TGF- β 1 ($P \geq 0.05$ vs. cells induced by TGF- β 1). The expression of Twist1 significantly decreased in tumor cells with Lv-miR-137 combined Lv-shRNA-PBC11 infection and TGF- β 1 induction. The protein detection data also showed that the expression of EMT factors N-cadherin and Akt in the downstream regulated by Twist1 were completely consistent with Twist1, while the expression of EMT inhibitor E-cadherin was opposite to Twist1 (**Fig.5C**).

Discussion

Thanks to the medical innovation and new drug development, the mortality rate of prostate cancer has declined in recent years, but its incidence rate is still increasing significantly worldwide. A statistic in 2018 shows that prostate cancer has become the most common male cancer in 20 countries and regions around the world, and the mortality of prostate cancer patients in five regions has risen to the second of tumor types [9–10]. Up to now, there is no specific treatment for prostate cancer. Postoperative metastasis and recurrence is an important reason for the increase of mortality in prostate cancer patients [11]. Under normal circumstances, metastasis is common in the middle and late stage of tumor progression, but different from other tumors, some patients with prostate cancer have been found to have tumor metastasis in the early stage of disease progression, and half of them have been found to have lymph node metastasis in the first diagnosis. Distal bone marrow metastasis is the most likely type of prostate cancer. More than 70% of prostate cancer patients will be accompanied by bone metastasis, and more than 85% of prostate cancer deaths are pathologically found to be complicated with tumor bone metastasis. However, so far, as no breakthrough has been made in the mechanism research and

treatment of prostate cancer metastasis, it has become an important research topic in urology to explore new targets for the treatment of prostate cancer, further study the potential molecular mechanism of malignant proliferation and metastasis of prostate cancer, and then provide scientific basis for the early diagnosis and prognosis of prostate cancer. AR (Androgen receptor signaling pathway, AR) is a key regulatory pathway for the occurrence and progression of prostate cancer. Studies have shown that it is closely related to the malignant progression and drug resistance of prostate cancer. AR and its downstream oncogenes have also been tried to be studied as targets for the treatment of pre prostate cancer. However, at present, drugs targeting these targets (such as bevacizumab) have not made significant clinical treatment progress [12–13].

In recent years, the basic research on prostate cancer has made continuous progress. The role of noncoding RNA in the progression of prostate cancer has been paid more and more attention and studied. MiRNAs (MicroRNA, miR) is a small, noncoding endogenous single stranded RNA that regulates gene expression by binding to the 3'-UTR of target genes (mRNAs), and participates in the processes of cell proliferation, differentiation, apoptosis and metabolism [14]. The expression of miRNAs is closely related to the occurrence and development of prostate cancer and has been proved to have the potential to diagnose and predict the prognosis of patients. The latest research shows that the contents of miR-215-5p, miR-93, miR-410-3p and miR-191 in prostate cancer tissues are higher than those in adjacent or normal prostate tissues, and their contents are positively correlated with clinical T stage, pelvic lymph node and bone metastasis. Compared with TNM stage, highly expressed miRNAs can provide additional median OS (overall survival, OS) information [15–18]. MiRNA is involved in prostate cancer progression and regulation in a variety of ways, and many miRNAs have been shown to inhibit prostate cancer progression. Bin Yang et al. reported that the level of miR-139-5p decreased in prostate cancer tissues and prostate cancer cell lines. The transfection of miR-139-5p mimics reduced the proliferation and migration of prostate cancer cells. The mechanism results also showed that miR-139-5p could inhibit the EMT progression of prostate cancer cells by inhibiting the expression of Sox5[19] Bin Yang et al. found that miR-34a was expressed in prostate cancer tissues, while overexpression of miR-34a could inhibit Wnt/ β -catenin pathway by regulating the transcriptional activity of Wnt1, which regulates the proliferation and migration of PC3 cells and promotes apoptosis [20]. Compared with normal/benign prostate tissues, miR-128 was proved to be significantly reduced in prostate cancer. Exogenous miR-128 inhibited tumor regeneration in a variety of prostate cancer xenotransplantation models. Researchers determined the tumor inhibitory function of miR-128 in prostate cancer by limiting the CSC characteristics mediated by BMI-1 and other central stem cell regulators which has potential significance for gene therapy of prostate cancer[21] MiR-137 is located on human chromosome 1, previous studies have shown that it is closely related to the development of prostate cancer. MiR-137 has been proved to be able to participate in the EMT regulation of prostate cancer cells exposed to hypoxic by targeting LGR4[22] Qi Quan Wu et al. have shown that miR-137 can effectively inhibit glycolysis in prostate cancer by down regulating NOX4, so it may be a potential theoretical target for the treatment of prostate cancer [23]. In addition, miR-137 was also confirmed to be related to the mechanism of drug resistance in prostate cancer. Prostate cancer is usually treated with hormones, including bicalutamide. Although this treatment was initially effective, the

tumor eventually gained resistance, resulting in CRPC recurrence. Preparation studies showed that methyl CpG binding protein 2 (MeCP2) and DNA methyltransferase (DNMT) synergistically promoted the methylation of miR-137 promoter, thereby reducing transcription, resulting in enhanced TRIM24 expression and glutamine metabolism. These findings describe a new mechanism affecting TRIM24 deregulation in human cancer and provide a molecular link between miR-137, TRIM24 and tumor proliferation in CRPC [24]. All these studies indicate that the decrease of hsa-miR-137 is the important reason for the progression of prostate cancer and its drug resistance which is consistent with our research data, and miR-137 has obvious tumor suppressor gene properties in the progression of prostate cancer metastasis. Compared with these studies, we found that hsa-miR-137 has lost its antitumor activity and cannot be attributed to the decrease in its content in metastatic prostate tumors. Specific research data showed that hsa-miR-137 lost negative regulation of the expression of its target gene Twist1, an prostate tumor metastasis promoting factor in metastatic tumors. So, what causes the functional inactivation of hsa-miR-137 in the progression of prostate cancer?

LncRNA (long noncoding RNA, lncRNA) directly targets and regulates DNA, RNA and protein targets by competing with endogenous RNA. Studies have shown that the expression of NEAT1 (nuclear rich transcription factor 1, NEAT1) and SNHG7 (small nucleolar RNA host gene 7, SNHG7) are increased in prostate cancer tissues, which are closely related to GS and TNM stages of prostate cancer, and the highly expressed lncRNA is an independent risk factor for the prognosis of prostate cancer patients [25–26]. With the development of transcriptome sequencing analysis, many lncRNAs have been found to be associated with prostate cancer [38,39]. PCA3 is a well-defined lncRNA, which has been approved by FDA for the clinical decision of repeated biopsy of prostate cancer. It is the first urine detection marker that can be used for the diagnosis of prostate cancer [27–28]. Studies have shown that PCA3 is not expressed in normal and malignant non prostate derived tissues, including urogenital organs such as kidney, bladder, seminal vesicle and testis. The expression of PCA3 increased only in patients diagnosed with prostate cancer, rather than BPH (benign prostatic hyperplasia, BPH), PIN (prostatic intraepithelial neoplasia, PIN), ASAP (atypical small acinar hyperplasia, ASAP), inflammation or chronic prostatitis [29–30]. These studies show that the functional study of lncRNA plays an important role in the clinical treatment and diagnosis of prostate cancer.

In this study, we found and reported for the first time that lncRNA-PBC11, which is closely related to the invasion and metastasis of prostate cancer, is located on chromosome 10 of human genome. PBC11 content in prostate cancer tissue is significantly higher than that in adjacent tissues, and is significantly higher in metastatic prostate cancer tissue than that in non-metastatic tumor tissue. Mechanism studies showed that PBC11 could inhibit the negative regulation of hsa-miR-137 on the expression of its target protein Twist1 in the form of miRNA sponge, which was completely consistent with the results of detection and correlation analysis of the expression of hsa-miR-137 and its target protein Twist1 in clinical samples. The data showed that the content of hsa-miR-137 and the expression of Twist1 protein maintained a negative correlation only in non-metastatic prostate cancer tissues, in metastatic prostate cancer tissues, although the content of hsa-miR-137 did not change significantly, the expression of

Twist1 was significantly up-regulated, and the two of them no longer had a negative correlation, indicating that hsa-miR-137 lost its antitumor activity in metastatic prostate cancer tissues.

In the functional experiment, the EMT model of prostate cancer cell was established by induction of TGF- β 1 *in vitro*, and the combined treatment scheme for suppression of EMT in prostate cancer cells was discussed with hsa-miR-137 and PBC11 as targets. The results showed that hsa-miR-137 overexpression and PBC11 silencing alone were not enough to effectively inhibit EMT induced by TGF- β 1 in prostate cancer cells, but hsa-miR-137 overexpression combined with PBC11 silencing showed a great inhibitory effect on EMT with the specific manifestation of the inhibition of proliferation, invasion and migration of prostate cancer cells under induced conditions by TGF- β 1. This is obviously the result of PBC11 knockdown releasing the inhibition of hsa-miR-137 on Twist1 expression, that is, restoring the operation of hsa-miR-137/Twist1, an inhibition pathway of prostate cancer invasion and metastasis. In short, benefit from knockdown of PBC11, hsa-miR-137 inhibited the expression of Twist1 and downstream proteins N-cadherin and AKT1(EMT promoting proteins), and up-regulated the expression of E-cadherin (EMT inhibitory protein) by the retrieve of anti-cancer function.

Our data also show that TGF- β 1 induction had no significant effect on the content of hsa-miR-137 in prostate cancer cells, but significantly increased the relative content of PBC11, indicating that PBC11 has the potential to become a clinical marker for screening the risk of invasion and metastasis of prostate cancer. Of course, the research on screening markers still needs a lot of attempts and innovation of detection methods, which will be an important direction and future works for us.

Declarations

Author contributions

Xuefeng Xie conceived the studies and designed the experiments. Haoyu Cui participated in the collection of clinical and experimental data, and carried out the biochemical experiments. Chuansheng Hou performed parts of the biochemical experiments. Qiang Ma and Zhuo Chen assisted in the collection of clinical data and analysis. Haoyu Cui and Xuefeng Xie wrote the manuscript with inputs from all other authors. All authors discussed and commented on the manuscript.

Competing interests: The authors declare no competing interests.

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Tables

Table 1
Clinicopathological features of 24 Prostate patients

Case	Age	metastatic site	TNM Staging
1	84	bone	T2bNxM1b
2	86	hepar & lung	T2bNxM1c
3	65	bone	T3bN1M1b
4	66	bone	T3bN0M1b
5	88	bone	T2bN0M1b
6	72	bone	T2cN0M1b
7	81	bone	T4NxM1b
8	83	lymph node & bone	T4N1M1b
9	79	bone	T4NxM1b
10	74	lymph node & bone	T2cN1M1b
11	70	lymph node & ureter	T4N1M1a
12	77	bone	T3bN0M1b
13	74	-	T2cN0M0
14	63	-	T2bN0M0
15	65	-	T2cN0M0
16	79	-	T2aN0M0
17	74	-	T2cN0M0
18	68	-	T2bN0M0
19	77	-	T2bN0M0
20	69	-	T3cN0M0
21	71	-	T3cN0M0
22	77	-	T2aN0M0
23	63	-	T2cN0M0
24	80	-	T2cN0M0

The prostate cancer staging system adopts the TNM staging system developed by the American Joint Committee on Cancer Staging (8th edition).

Figures

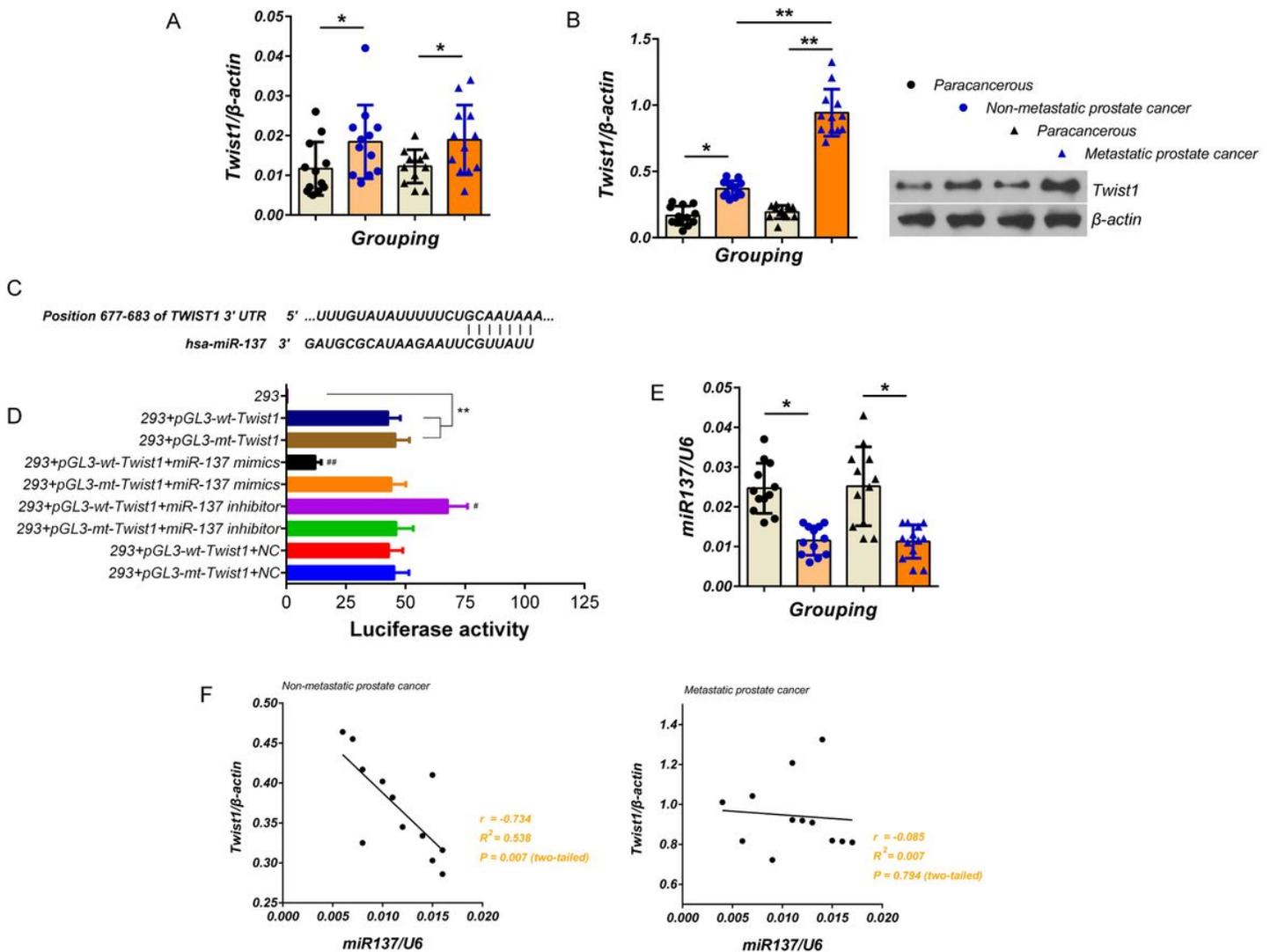


Figure 1

Abnormal role of negative regulation of Twist 1 by has miR-137 in metastasis progression prostate cancer

(A) Relative mRNA levels of Twist1 in metastatic and non-metastatic tumors (n = 12) were analyzed using RT-qPCR. β -actin served as an internal reference. The $2^{-\Delta Ct}$ method was used to analyze inter-group differences. *, $p < 0.05$, and **, $p < 0.01$. (B) Twis1 protein level in 24 prostate tumors. β -actin (43 kDa) served as an internal reference. *, $p < 0.05$, and **, $p < 0.01$. (C) Predicted binding site of hsa-miR-137 in 3'-UTR of Twist1. (D) The intracellular luciferase activity of 293 cells was detected 48 hours after co-transfection. The histogram shows the relative luciferase activity, and the abscissa represents the co-transfection of different combinations. **, $p < 0.01$ vs 293 cells; # $p < 0.05$, ## $p < 0.01$ vs 293 cells transfected with pGL3-wt-Twist1 or pGL3-mt-Twist1. (E) Relative levels of hsa-miR-137 in metastatic and non-metastatic tumors (n = 12) were analyzed using RT-qPCR. U6 served as an internal reference. *, $p <$

0.05, and **, $p < 0.01$. (F) Correlation analysis of the hsa-miR-137 and Twist1 protein in metastatic and non-metastatic tumors. All data are expressed as mean \pm SD.

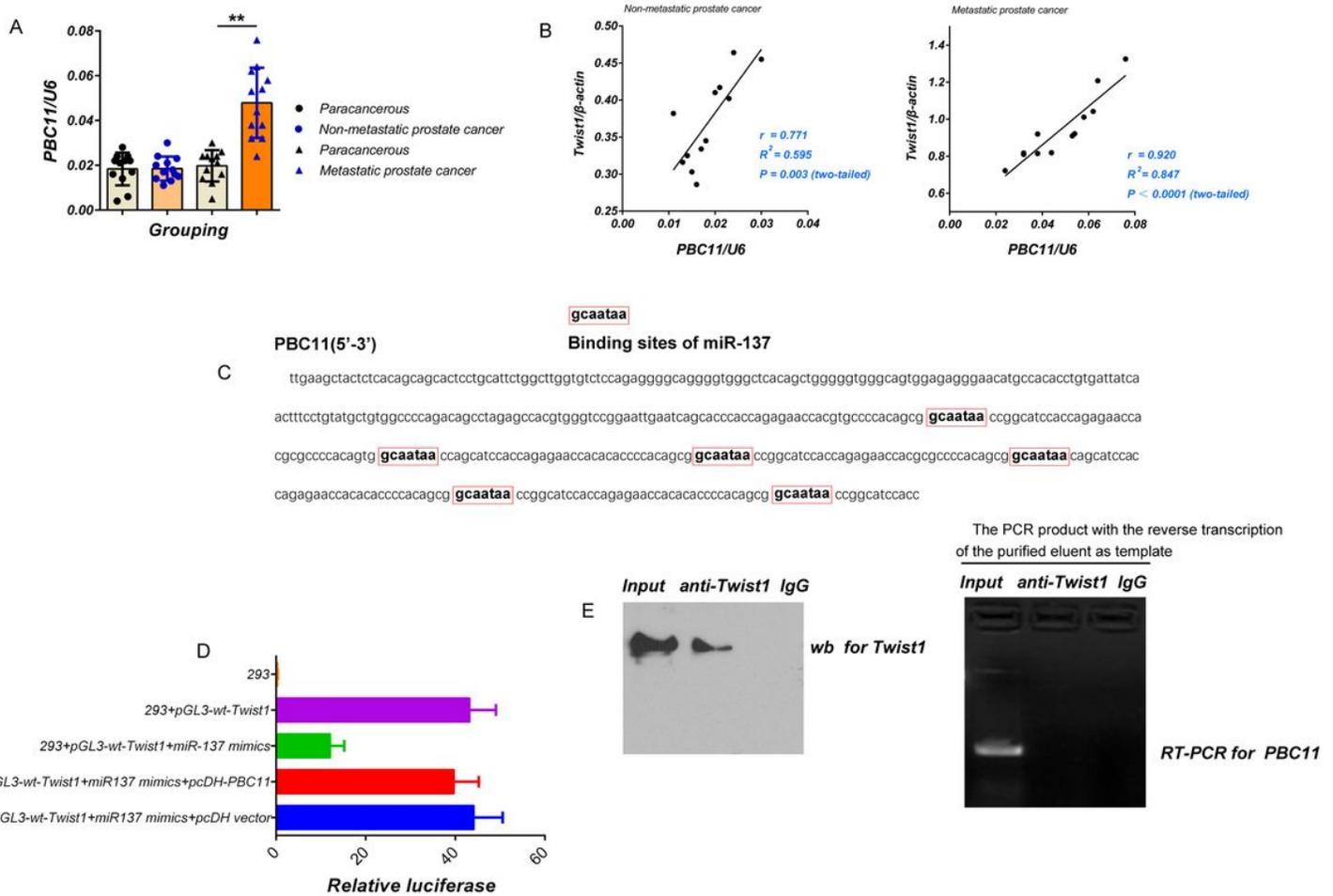


Figure 2

Abnormal of negative regulation of Twist1 by has miR-137 in metastasis progression prostate cancer

(A) Relative levels of PBC11 in metastatic and non-metastatic prostate tumors (n=12) were analyzed using RT-qPCR. U6 served as an internal reference. The $2^{-\Delta Ct}$ method was used to analyze inter-group differences. **, $p < 0.01$. (B) Correlation analysis of the PBC11 and Twist1 protein in metastatic and non-metastatic prostate tumors (n=12). (C) Bioinformatics analysis of the binding sites of hsa-miR137 on PBC11. (D) The luciferase activity of 293T cells was detected 48 hours after co-transfection. **, $p < 0.01$. (E) RIP assay. Immunoprecipitation of Twist1 (left), agarose gel electrophoresis for PCR amplification of the predicted Twist1 binding sequence in PBC11(right). All data are expressed as mean \pm SD of at least three independent experiments.

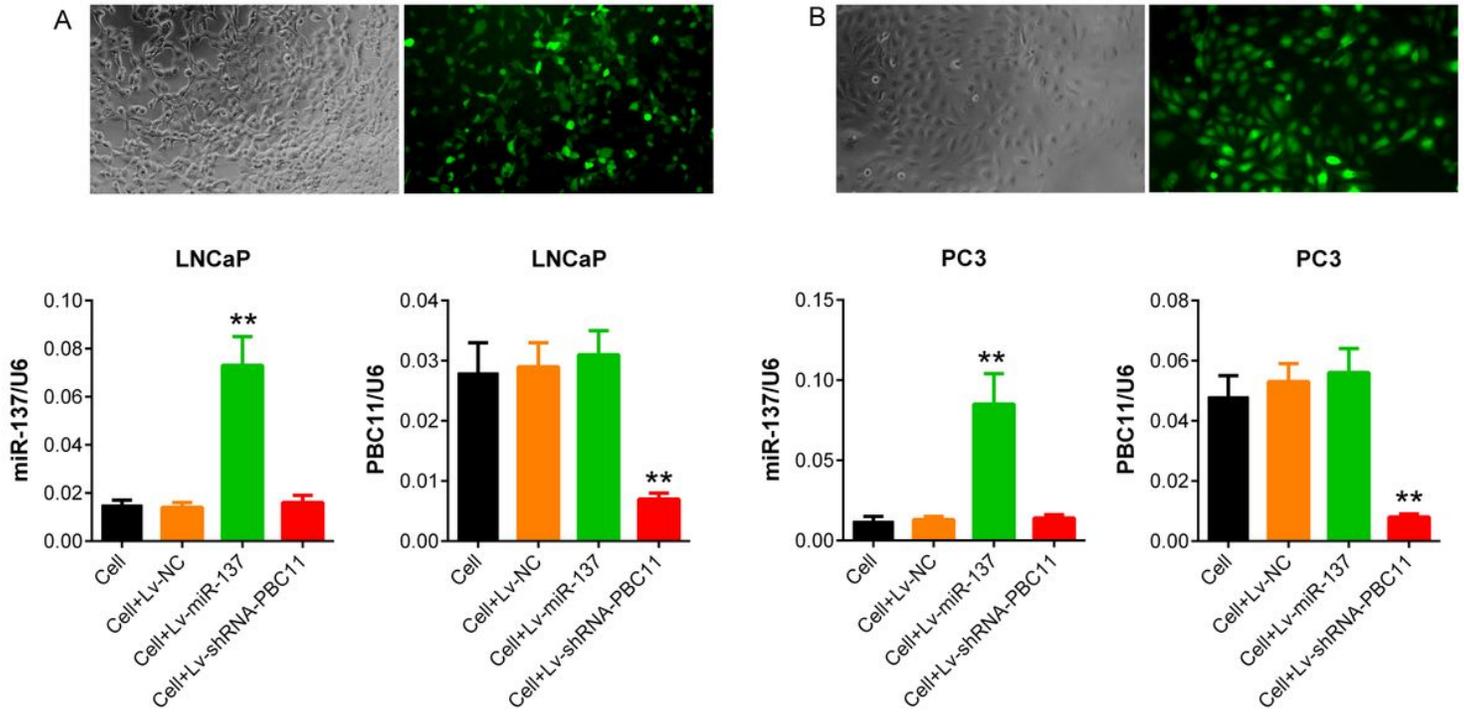


Figure 3

Lentivirus mediated has-miR-137 overexpression and PBC11 silencing in prostate cancer cells

(A) Virus infection efficiency analysis. GFP expression 72 h following LNCaP and PC3 cells infection with Lv-miR-137 is presented. (B) Analysis of intervention efficiency. Hsa-miR137 and PBC11 levels were determined by RT-qPCR with U6 as internal controls. Each experiment was performed in triplicate and data are expressed as the mean \pm standard deviation. ** $P < 0.01$. vs the other three groups.

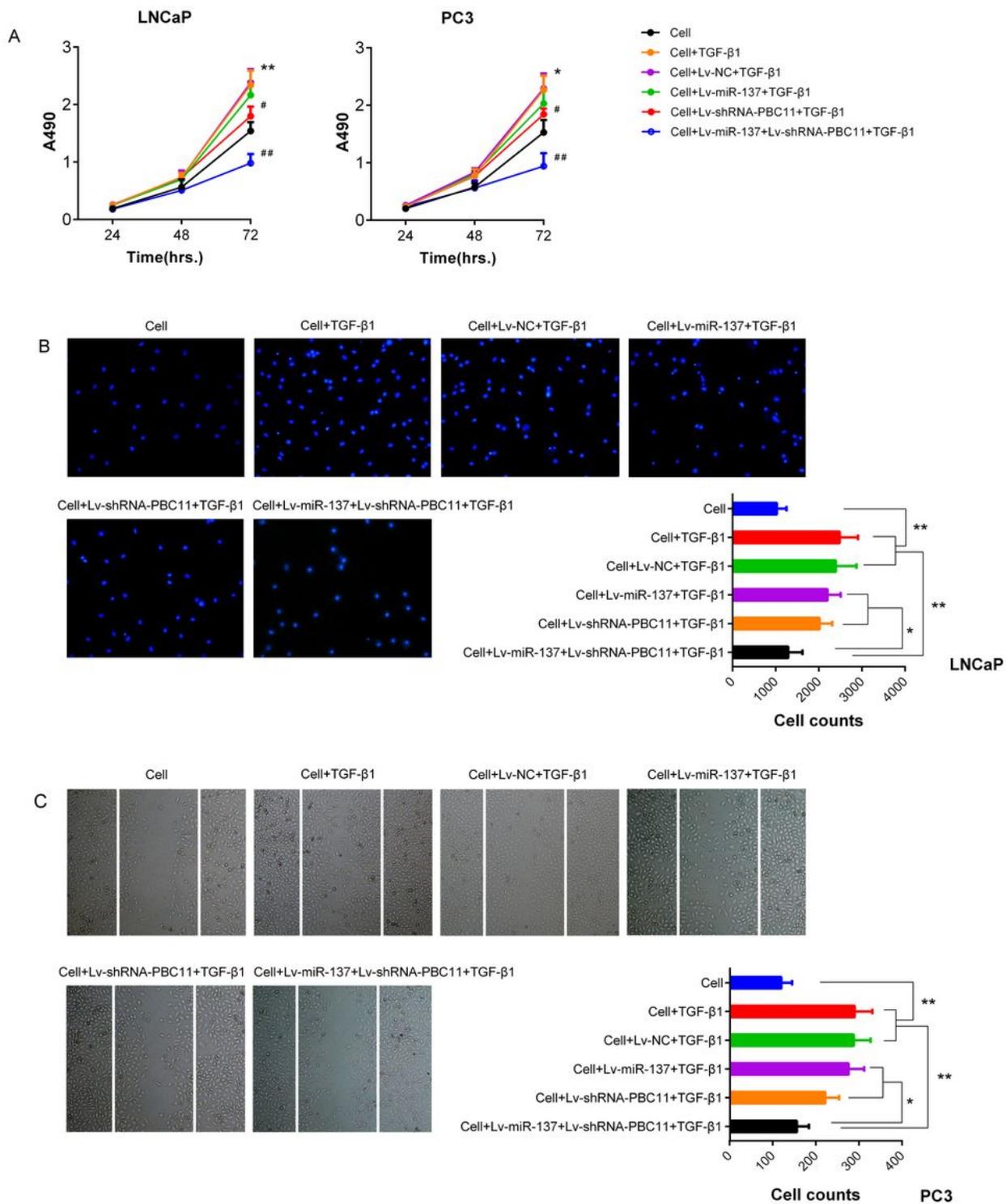


Figure 4

Effect of overexpression of has-miR-137 and knockdown of PBC11 on cell Proliferation, Invasion and Migration in LNCaP and PC3 cells

(A) Proliferation of cells was determined using CCK-8 assay. The x-coordinate represents the cell grouping and the y-coordinate represents the absorbance at 490 nm. (B) Invasion of LNCaP cells was determined

using a trans-well assay. DAPI was used to stain the cells that passed through the membrane, and the cell counts were estimated based on the absorbance and a standard curve. The x-coordinate represents cell grouping and the y-coordinate represents the cell number. (C) Migration of PC3 cells was determined by scratch wound healing assay. Images of cells were used to observe the number of cells growing across the scratched lines. The number of cells growing across the scratched line was defined as the number of migrated cells, and used to compare the changes in the migration ability in each group. The x-coordinate represents cell grouping and the y-coordinate represents the cell number. ** $p < 0.01$, * $p < 0.05$ (t-test). The tests were carried out in biological triplicates, and data are expressed as the mean \pm SD.

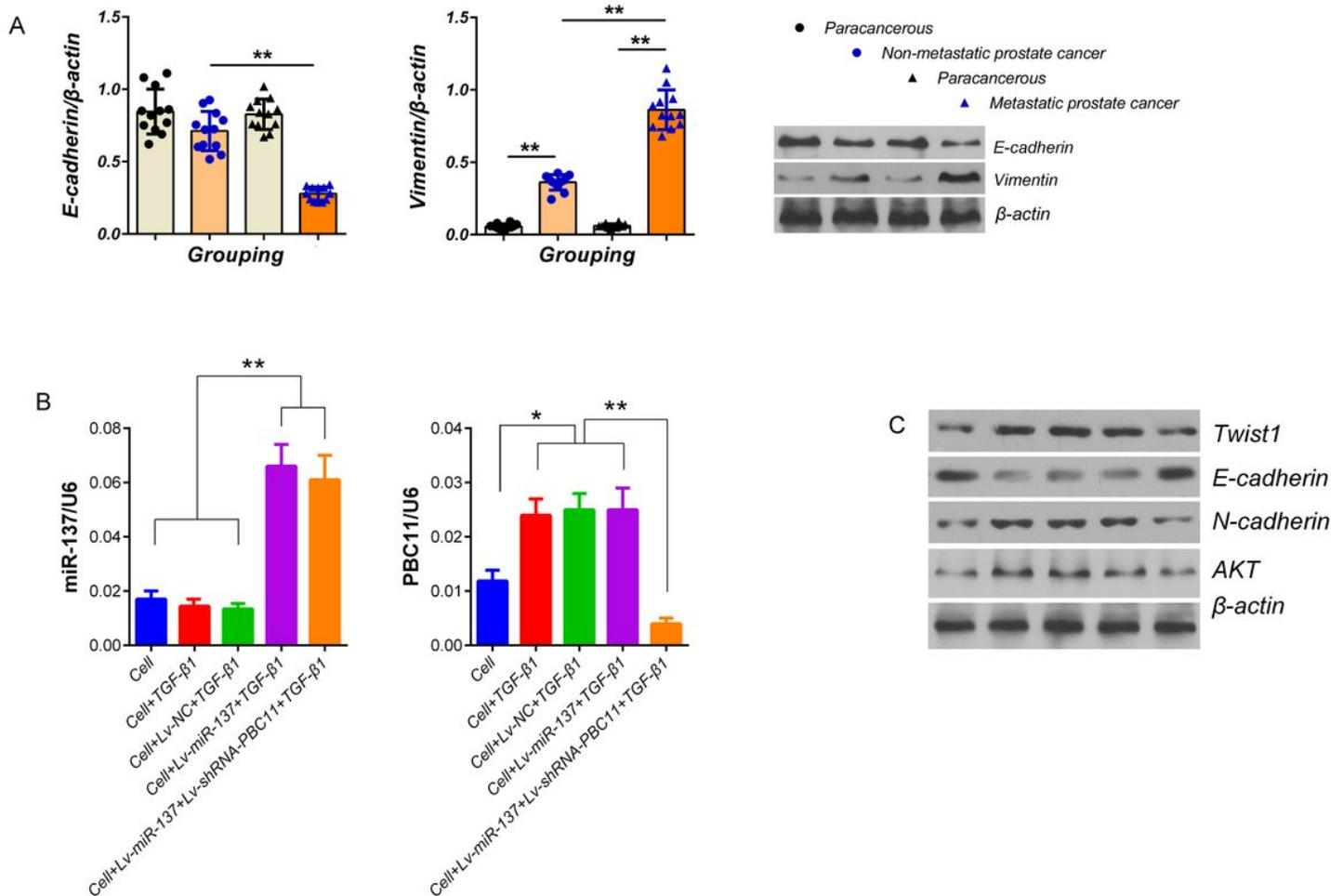


Figure 5

Analysis of the effect of PBC11 knockdown on EMT progression regulated by miR-137/Twist1 pathway in prostate cancer cells induced by TGF- β 1

(A) Expression of E-cadherin and Vimentin in 24 pairs of prostate tumors and their precancerous lesions were detected by western blotting with β -actin served as internal references. (B) Relative levels of hsa-miR-137 and PBC11 were detected by RT-qPCR assay by using the $2^{-\Delta Ct}$ method with U6 were used as internal control. (C) Western blotting for expression of Twist1, E-cadherin, N-cadherin and AKT1 proteins in

LNCaP cells with different treatments. β -actin served as an internal reference. All the data that are expressed as the mean \pm standard deviation (SD). ** $p < 0.01$, * $p < 0.05$ (t-test).