CD147 promotes epithelial-mesenchymal transition of prostate cancer cells via the Wnt/β-catenin pathway

FANG FANG[1*], QIANG LI[1*], MINGYUE WU[1], CHUNHUA NIE[1], HAIYUE XU[2] and LIGUO WANG[3]

[1]Department of Immunology, Jilin Medical University; [2]Department of Immunology, Institute of Medicine, Yanbian University, Yanji, Yanbian, Jilin 133002; [3]Department of Urology Surgery, Affiliated Hospital of Jilin Medical University, Jilin City, Jilin 132013, P.R. China

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Abstract. The majority of deaths among patients with prostate cancer (PCa) occur following metastasis; therefore, there is a critical need for effective treatment of metastatic PCa. Epithelial-mesenchymal transition (EMT) is vital in the early stage of cancer cell metastasis and CD147 has been reported to be associated with various types of cancer. The goal of this study was to investigate the role of CD147 in the EMT of PCa cells via short hairpin (sh)RNA-mediated knockdown of CD147 in lymph node carcinoma of the prostate (LNCaP) cells. Reverse transcription-quantitative PCR and western blotting were performed to examine gene and protein expression. Cell migration and invasion were detected using a Transwell assay. Cell Counting Kit-8 assay was performed to investigate cell viability. The knockdown of CD147 in LNCaP cells (LNCaP/shCD147 cells) resulted in an increase in the expression of E-cadherin (an epithelial marker), and a decrease in the expression of N-cadherin and vimentin (mesenchymal markers). Importantly, the downregulation of CD147 in LNCaP cells inhibited the expression levels of nuclear β-catenin and Snail, and phosphorylation of glycogen synthase kinase (GSK)-3β on Ser 9, and increased the expression of phosphorylated (p)-β-catenin (Ser33/37/Thr41). Treatment with lithium chloride (LiCl), a Wnt/β-catenin pathway agonist or a GSK-3β inhibitor, attenuated CD147 downregulation-induced p-β-catenin (Ser33/37/Thr41) expression, which resulted in the upregulation of β-catenin in the nucleus. LiCl treatment prompted β-catenin-mediated expression of target proteins such as Snail and vimentin in LNCaP/shCD147 cells, and prevented E-cadherin expression, a molecule downstream to Snail. In conclusion, these findings revealed an important role of CD147 in the regulation of the invasive and metastatic potential of PCa cells. CD147, via modulation of the Wnt/β-catenin pathway, may be implicated in the regulation of EMT of PCa cells and could be a potential therapeutic target for PCa.

Correspondence to: Dr Liguo Wang, Department of Urology Surgery, Affiliated Hospital of Jilin Medical University, 5 Jilin Street, Fengman, Jilin City, Jilin 132013, P.R. China
E-mail: urolancet@sina.com

*Contributed equally

Abbreviations: PCa, prostate cancer; EMT, epithelial-mesenchymal transition; LNCaP, lymph node carcinoma of the prostate; LiCl, lithium chloride

Key words: CD147, prostate cancer, EMT, metastasis, Wnt/β-catenin pathway

Introduction

Prostate cancer (PCa) remains the most prevalent cancer in men in North America and the second leading cause of cancer-related mortality in males. The majority of PCa-related deaths are due to metastases rather than primary tumour burden (1,2). PCa can metastasize to the bone, lymph nodes, liver, adrenal glands or lungs (3). The 5-year survival rate of patients with non-metastatic PCa has been reported to be 98.9% but that of patients with metastatic PCa is only 28.2% (4).

Epithelial-mesenchymal transition (EMT) is a differentiation program where cells switch from epithelial to mesenchymal phenotypes, which serves a key role in the early stage of cancer cell metastasis (5). EMT is initiated following the dissolution of tight junctions resulting in the loss of apical-basal cell polarity. The characteristics of EMT include altered expression of proteins, coupled with the upregulation of mesenchymal markers, such as N-cadherin and vimentin, and loss of epithelial markers, such as E-cadherin (6). The Wnt/β-catenin signalling pathway has an essential role in EMT, in which β-catenin functions as a key signalling mediator. In epithelial tissues under non-cancerous conditions, β-catenin participates in the linking of E-cadherin and contributes to cell-cell adhesion (7). During EMT, β-catenin dissociates from the E-cadherin/β-catenin complex at the cell membrane, accumulates in the cytoplasm and translocates into the nucleus where it acts as a transcriptional activator to promote the transcription of EMT-related genes, such as Snail, vimentin and matrix metalloproteinase-7 (8). Numerous studies have demonstrated that EMT is a critical process in the invasion and metastasis of PCa (9,10). EMT has been shown to occur after androgen withdrawal therapy in PCa and is associated with a poor clinical prognosis (11). Therefore, it is important to identify the molecular triggers of EMT in

order to decrease metastasis and improve the survival rates of patients with PCa.

CD147, also named extracellular matrix metalloproteinase inducer, is a glycosylated transmembrane member of the immunoglobulin superfamily. CD147 is highly expressed on the cell surface of most cancer cells, including PCa cells (12). During tumorigenesis, CD147 contributes to cell metastasis, drug resistance and angiogenesis (13-15). A previous study demonstrated that CD147 plays a vital role in the invasion and metastasis of PCa cells (13). However, the relationship between CD147 and EMT in PCa cells remains elusive. The aim of the present study was to evaluate the role of CD147 in induction of EMT and to decipher the underlying molecular mechanisms.

Materials and methods

Cell culture. Androgen-sensitive LNCaP cells, (provided by the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences) infected with the pGV248 lentiviral vector as the backbone for the CD147 short hairpin (sh)RNA construct (Shanghai GeneChem Co., Ltd) were termed LNCaP/shCD147 cells. LNCaP/Scramble cells (negative control) were established by infecting LNCaP cells with the pGV248 lentiviral vector containing a control shRNA sequence. The target sequences for the CD147 and control shRNA duplexes were 5'-GTCGTCAGAACACATCAACT-3' and 5'-CAGTCGCGTTTGCGACTGG-3', respectively (16). Lentiviral infection was performed following the manufacturer's protocol (Shanghai GeneChem Co., Ltd). Briefly, $1x10^6$ cells/well were seeded in 12-well plates 24 h prior to the experiment. The cells were infected at 30 multiplicity of infection. Lentiviral vectors were added in the presence of polybrene (5 μg/ml) and the supernatant was removed 24 h post-infection. The cells were maintained in RPMI 1640 medim (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in an atmosphere of 95% air and 5% CO_2. At 3 days after lentivirus infection, cells were digested by trypsin and collected by centrifugation (1,200 x g at room temperature for 10 min), and reverse transcription-quantitative PCR (RT-qPCR) was used to determine the expression of CD147 gene levels in the cells of the two groups. For western blotting experiments, cells were treated with 20 mM lithium chloride (LiCl; cat. no. L9650; Sigma-Aldrich; Merck KGaA) for 3 h at 37˚C.

RT-qPCR. Total RNA was extracted with TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA was synthesized using 1 μl RNA in 30 μl of reaction buffer by RT using an All-in-One First-Strand cDNA Synthesis kit (cat. no. QP006; GeneCopoeia Inc.) and oligo(dT) 18 primers at 42˚C for 90 min according to the manufacturer's instructions. RT-qPCR was performed using an UltraSYBR Mixture with ROX (CoWin Biosciences) according to the manufacturer's instructions on the ABI 7500 fluorescence qPCR instrument (Applied Biosystems). The following primer pairs were used for the qPCR: CD147 forward, 5'-CAGAGTGAAGGCTGTGAAGTCG-3' and reverse, 5'-TGCGAGGAACTCACGAAGAA-3' and β-actin forward, 5'-CACTGTGCCCATCTACGAGG-3' and reverse, 5'-TAATGTCACGCACGATTTCC-3'. The reaction conditions were as follows: 2 min at 50˚C and 10 min at 95˚C, followed by 40 cycles (15 sec at 95˚C, 30 sec at 60˚C and 30 sec at 70˚C) and final extension at 72˚C for 30 sec. All values were normalized to β-actin expression. Relative quantification was performed using the $^{\Delta\Delta Cq}$ method, and the results are expressed in a linear form using the formula $2^{-\Delta\Delta Cq}$ (17).

Cell Counting Kit-8 (CCK-8) assay. The cells ($1x10^4$/well) were seeded in 96-well plates and cultured at 37˚C with 5% CO_2 for 2, 4, 6 and 8 days. Cell viability was assessed using CCK-8 (Beyotime Institute of Biotechnology). After 2, 4, 6 and 8 days, 10 μl CCK-8 solution was added to the medium, the supernatants were removed and the absorbance of the sample was measured at 450 nm. Cell viability (%)=(experimental group/control group) x100.

Transwell assay. The cell migration and invasion assays were performed in 24-well plates with a filter chamber (pore size, 8 μm; Corning Inc.). For the migration assays, the cells ($5x10^4$/well, 200 μl) were seeded into the upper chamber without a Matrigel-coated membrane with RPMI-1640 medium containing 0.1% FBS. The bottom chambers were filled with 600 μl RPMI-1640 medium containing 10% FBS. For the invasion assay, the cells ($1x10^5$/well, 200 μl) were seeded into the upper chamber on top of a Matrigel-coated membrane with RPMI-1640 medium containing 0.1% FBS. Following incubation for 48 h at 37˚C, the remaining cells in the upper chamber were gently removed with a cotton swab. The cells on the lower surface of the membrane filter were stained with 0.1% crystal violet for 20 min at room temperature and observed by light microscopy (magnification, x200; Olympus Corporation).

Western blotting. The cells were washed twice with PBS and then lysed with lysis buffer (Beyotime Institute of Biotechnology) containing a cocktail of protease inhibitors (Roche Diagnostics GmbH). To extract specific protein compartments, the Compartmental Protein Extraction kit (Beyotime Institute of Biotechnology) was used, according to the manufacturer's protocol. Protein concentration was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein (30 μg) were separated on 10% sodium dodecyl sulphate-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (EMD Millipore). The membranes were then blocked with 5% nonfat milk in PBS containing 0.05% Tween-20 at room temperature for 2 h. The membranes were incubated with rabbit monoclonal antibodies against CD147 (cat. no. 13287), human p-glycogen synthase kinase (GSK)-3β (Ser 9; cat. no. 9322), GSK-3β (cat. no. 9315), E-cadherin (cat. no. 3195), N-cadherin (cat. no. 13116), vimentin (cat. no. 5741), β-catenin (cat. no. 8480), p-β-catenin (Ser 33/37/Thr 41; cat. no. 9561) and Snail (cat. no. 3879) all at 1:1,000 dilution (Cell Signaling Technology, Inc.) overnight at 4˚C. β-actin (cat. no. 4970) and lamin B (cat. no. 13435) were used as loading controls (both 1:2,000; Cell Signaling Technology, Inc.). Subsequently, the membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:3,000; cat. no. A0208; Beyotime Institute of Biotechnology) and was visualized and quantified using

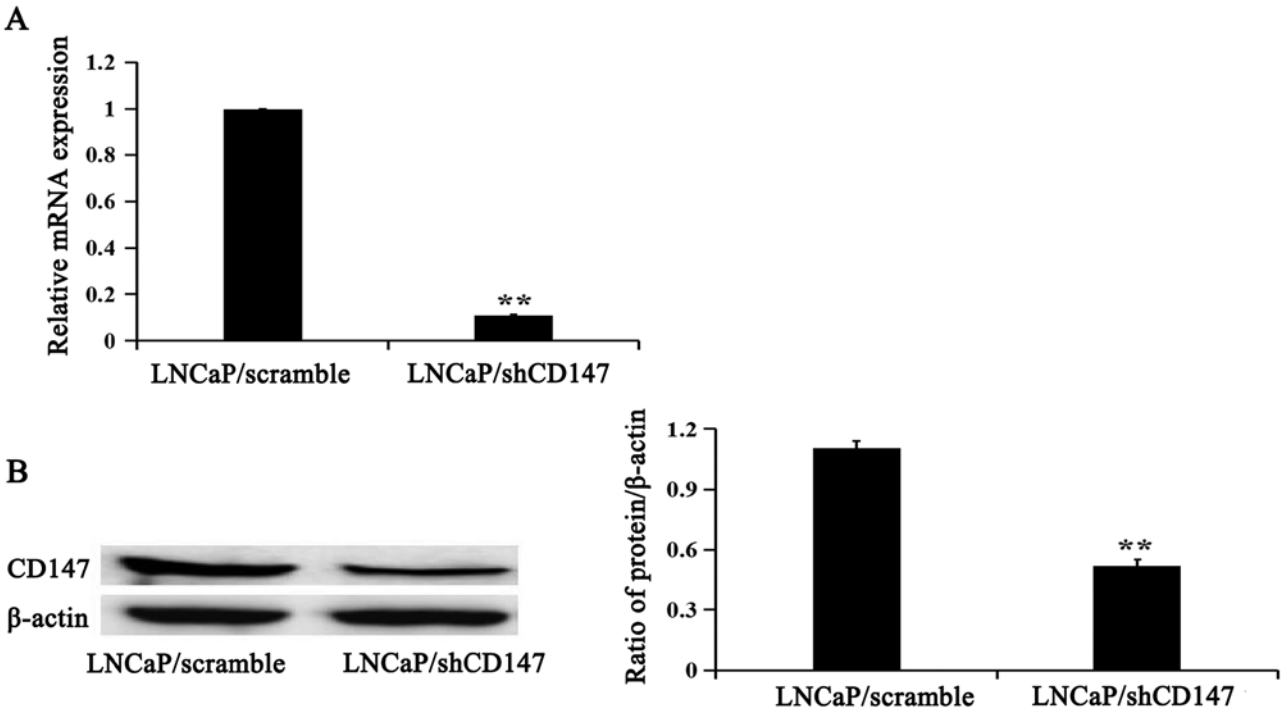

Figure 1. mRNA and protein expression levels of CD147 in LNCaP cells depleted of CD147 as detected by reverse transcription-quantitative polymerase chain reaction and western blotting. (A) mRNA and (B) protein expression in LNCaP/Scramble and LNCaP/shCD147 cells. Values are presented as the mean ± standard deviation of three experiments. **P<0.01 vs. LNCaP/Scramble. LNCaP, lymph node carcinoma of the prostate; sh, short hairpin RNA.

an enhanced chemiluminescence detection system (iBright CL1500; Invitrogen; Thermo Fisher Scientific, Inc.).

Statistical analysis. Data are expressed as the mean ± standard deviation of three experiments. Differences between two groups were assessed using the Mann-Whitney U test and comparisons between multiple groups were performed using the Kruskal-Wallis test followed by Dunn's multiple comparisons post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CD147 expression in LNCaP/Scramble and LNCaP/shCD147 cells. To study the function of CD147, the LNCaP cell line, which has cytological features of prostate cancer cells, was chosen to knock down CD147 expression. RT-qPCR and western blotting were performed to evaluate the relative mRNA and protein expression levels. As shown in Fig. 1, the mRNA and protein expression levels of CD147 were depleted in LNCaP cells following infection with lentiviruses to deplete CD147 expression (LNCaP/shCD147) compared with LNCaP cells infected with the control lentivirus (LNCaP/Scramble).

CD147 promotes the growth of LNCaP cells. The cells were cultured for 2, 4, 6 and 8 days with cell growth measured using the CCK-8 assay. As shown in Fig. 2, knockdown of CD147 expression in LNCaP cells inhibited cell growth compared with LNCaP cells infected with the scramble lentivirus. LNCaP cells were infected with the lentiviral shCD147 or scramble vector for 3 days and further experiments were conducted

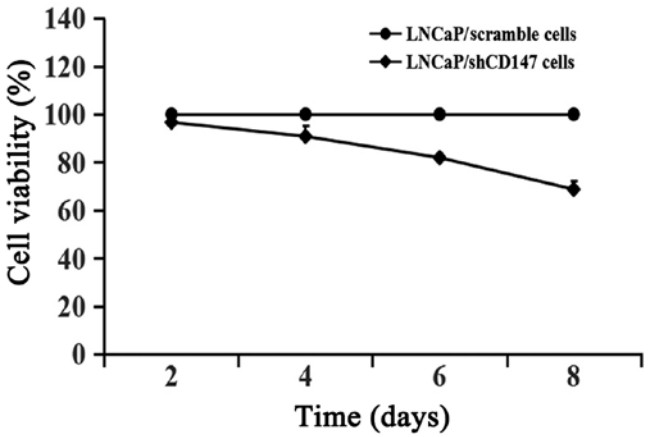

Figure 2. CD147 promotes LNCaP cell viability. LNCaP/shCD147 and LNCaP/Scramble cells were seeded in 96-well plates and incubated for 2, 4, 6 or 8 days. Cell viability was determined using a Cell Counting Kit-8 assay. Values are normalized to LNCaP/Scramble and presented as the mean ± standard deviation of three experiments. LNCaP, lymph node carcinoma of the prostate; sh, short hairpin RNA.

3 days after infection in order to reduce the influence of depletion of CD147-induced cell growth inhibition.

CD147 promotes the migration and invasion of LNCaP cells. To investigate whether CD147 inhibits the migration and invasion of LNCaP cells, Transwell assays were performed with un-coated and Matrigel-coated membranes to determine the effects of CD147 expression on cell migration (Fig. 3A) and invasion (Fig. 3B). The results showed that the cells which had been depleted of CD147 expression were significantly less

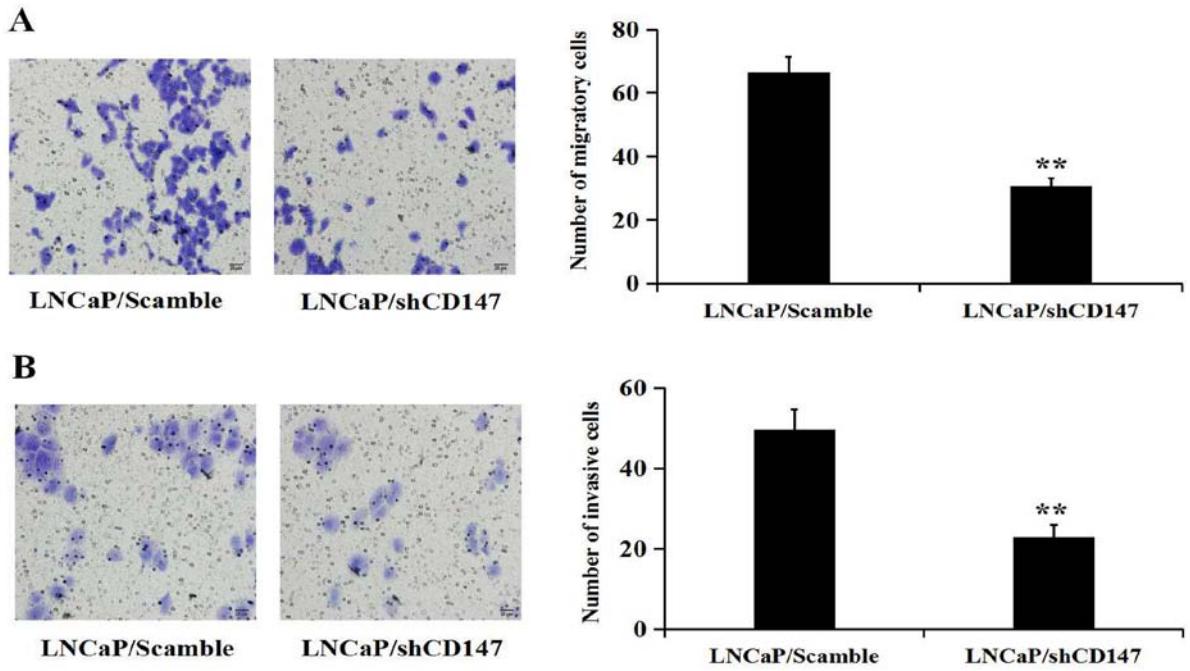

Figure 3. CD147 promotes the migration and invasion of LNCaP cells. Effect of CD147 knockdown on (A) migration and (B) invasion of LNCaP cells. Scale bars, 20 μm. Values shown are presented as the mean ± standard deviation of three experiments. **P<0.01 vs. LNCaP/Scramble. LNCaP, lymph node carcinoma of the prostate; sh, short hairpin RNA.

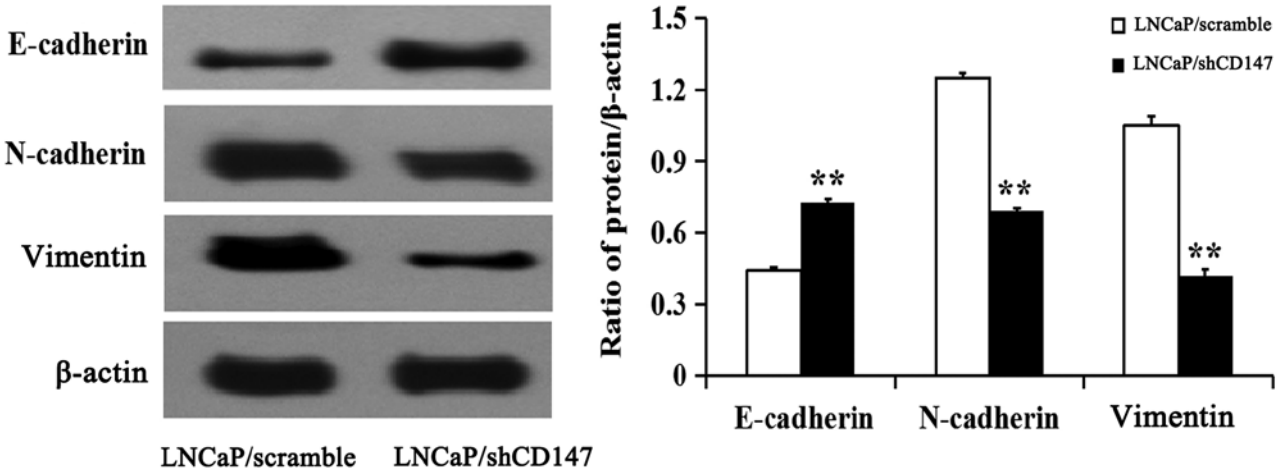

Figure 4. CD147 promotes epithelial-mesenchymal transition of LNCaP cells. The expression of the epithelial marker E-cadherin and the mesenchymal markers N-cadherin and vimentin was determined in LNCaP/Scramble and LNCaP/shCD147 cells by western blotting. Values shown are presented as the mean ± standard deviation of three experiments. **P<0.01 vs. LNCaP/Scramble. LNCaP, lymph node carcinoma of the prostate; sh, short hairpin RNA.

migratory or invasive than the LNCaP cells that had been infected with the Scramble lentivirus.

CD147 induces EMT in LNCaP cells. To assess whether CD147 affects the EMT of PCa cells, the expression of key markers of EMT was detected. The results demonstrated that the knockdown of CD147 in LNCaP cells led to an increase in the expression of the epithelial marker E-cadherin, and a decrease in the expression of mesenchymal markers N-cadherin and vimentin compared with the LNCaP/Scramble cells (Fig. 4). These results suggested that CD147 could promote the EMT of LNCaP cells.

CD147-induced EMT is associated with activation of the Wnt/β-catenin pathway. The Wnt/β-catenin pathway is the one of the key pathways that induce EMT. Therefore, the expression levels of the Wnt pathway components β-catenin and GSK-3β were analysed in LNCaP cells. It was revealed that knockdown of CD147 significantly inhibited the nuclear expression of β-catenin and Snail (P<0.05; Fig. 5A). Knockdown of CD147 in LNCaP cells also reduced the expression of p-GSK-3β (Ser9) (P<0.05; Fig. 5B). Subsequently, the present study sought to verify whether CD147 participated in the EMT process through the Wnt/β-catenin signalling pathway. To address this question, LNCaP/Scramble and LNCaP/shCD147

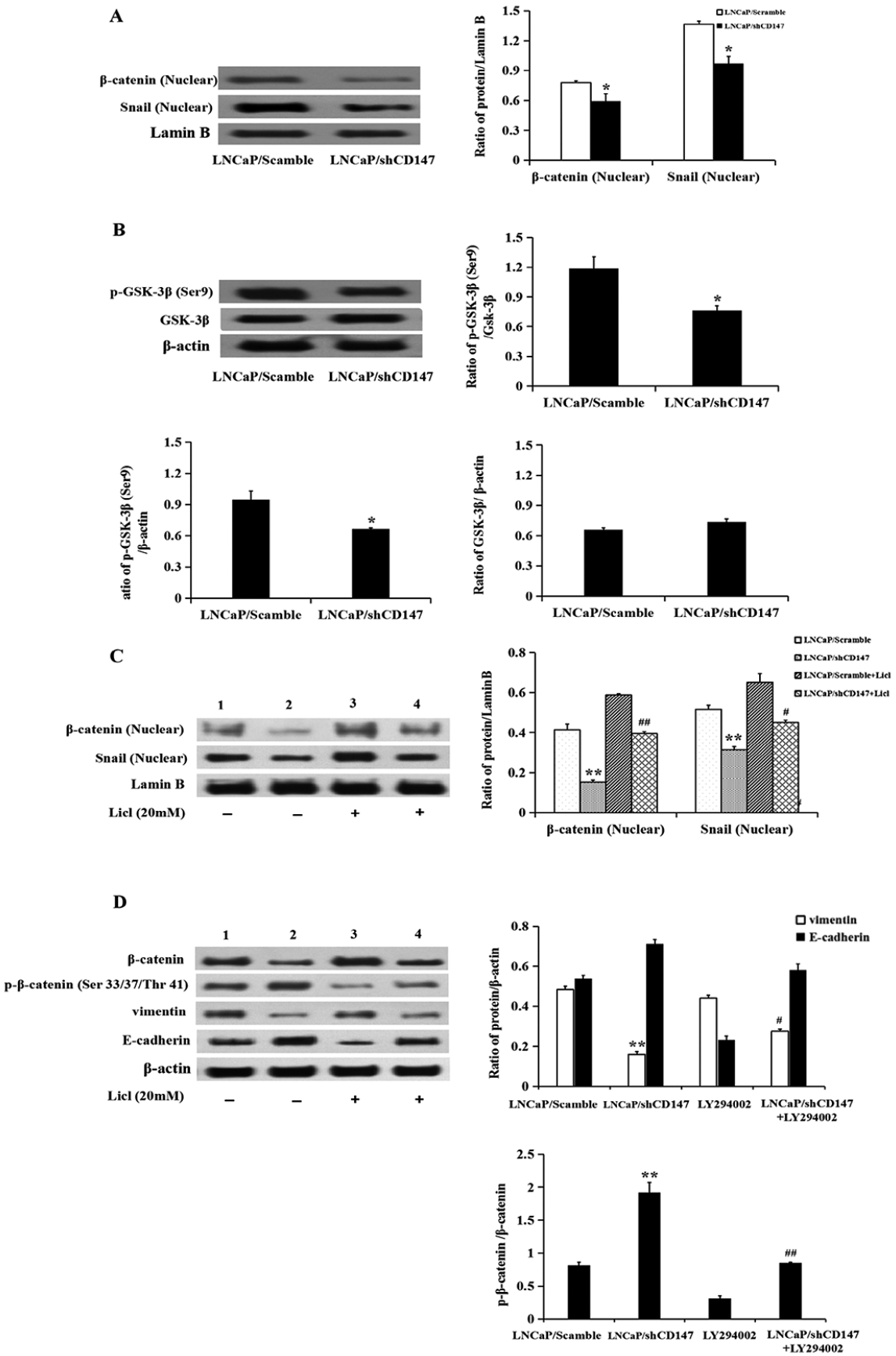

Figure 5. CD147 regulates epithelial-mesenchymal transition via the Wnt/β-catenin pathway. (A and B) Expression of β-catenin, Snail and p-GSK-3β (Ser9) was determined in LNCaP/Scramble and LNCaP/shCD147 cells by western blotting. (C and D) Expression of β-catenin, Snail, p-β-catenin (Ser33/37/Thr41), vimentin and E-cadherin following treatment with or without LiCl in LNCaP/Scramble and LNCaP/shCD147 cells by western blotting. Values are presented as the mean ± standard deviation of three experiments. Lanes 1 and 3, LNCaP/Scramble group; Lanes 2 and 4, LNCaP/shCD147 group. *P<0.05, **P<0.01 vs. LNCaP/Scramble; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. LNCaP/shCD147. LNCaP, lymph node carcinoma of the prostate; LiCl, lithium chloride; sh, short hairpin RNA; p-, phosphorylated; GSK-3β, glycogen synthase kinase-3β.

cells were treated with lithium chloride (LiCl) for 3 h. LiCl, as an agonist of the Wnt/β-catenin signalling pathway and a GSK-3β inhibitor, did not affect the expression of CD147(data not shown). The expression levels of β-catenin, p-β-catenin (Ser 33/37/Thr 41), E-cadherin and β-catenin-targeted proteins, including Snail and vimentin, were subsequently examined. The expression of p-β-catenin (Ser33/37/Thr41) was upregulated in LNCaP/shCD147 cells, as expected, which was consistent with the downregulation of β-catenin, Snail and vimentin. Treatment with LiCl significantly attenuated the upregulation of p-β-catenin (Ser33/37/Thr41) expression that followed knockdown of CD147, leading to an increase in β-catenin, Snail, and vimentin expression. As E-cadherin is downstream of Snail, the change in E-cadherin expression was consistent with the change in Snail expression (Fig. 5C and D).

Taken together, these data suggested that knockdown of CD147 may induce inhibition of the Wnt/β-catenin pathway, leading to the activation of GSK-3β, degradation of β-catenin, and subsequent downregulation of Snail and vimentin.

Discussion

Tumour metastasis is a crucial cause of treatment failure and mortality in patients with cancer. Cancer morbidity and mortality are largely related to the spread of the primary, localized tumour to adjacent and distant sites, which result in the arrival of malignant cells to their growth and proliferation in the host organ (18). Some studies have shown that tumour epithelial cells lose epithelial cell polarity and gain mesenchymal morphology, promoting tumour metastasis (6,19). CD147 serves as a pro-survival and pro-migration factor in physiological and pathological processes, and it has been reported to promote invasion and EMT in colorectal cancer by regulating the MAPK/ERK signalling pathway (20). Moreover, CD147 was able to increase hypoxia-induced metastasis and EMT in oesophageal cancer cells by regulating hypoxia-inducible factor-1α (21), and promoted transforming growth factor-β-induced EMT and invasion via Snail and Slug in hepatocellular carcinoma (22). In this study, it was found that downregulation of CD147 inhibited the invasion and migration of PCa cells. The study then sought to characterize the contribution of CD147 in the control of EMT-driven metastasis in PCa cells. One of the most distinctive features of EMT is the loss of E-cadherin expression. In this study, the knockdown of CD147 upregulated the expression of the epithelial marker E-cadherin, and downregulated the expression of mesenchymal markers N-cadherin and vimentin in LNCaP cells, suggesting that CD147 may promote EMT in LNCaP cells.

The Wnt/β-catenin signalling pathway is also associated with the EMT process. The increased levels of β-catenin lead to its nuclear translocation and activation of its target genes, such as the EMT-related genes Snail and vimentin. Snail, a target of β-catenin, is a critical transcription factor in the regulation of EMT. In this process, Snail causes the transcriptional repression of E-cadherin by the assembly of the repressor complex at the E-cadherin promoter (23). In this study, one of the significant findings was that of the role of CD147 in EMT was dependent on the Wnt/β-catenin signalling pathway. The study revealed that knockdown of CD147 led to β-catenin downregulation in the nucleus, and decreased expression of Snail and vimentin. In the Wnt/β-catenin pathway, GSK-3β is an upstream factor of β-catenin, which promotes its phosphorylation at Ser33/37and Thr41. The phosphorylated form of β-catenin is ubiquitinated through E3 ubiquitin ligase and consequently targeted for ubiquitin-mediated degradation, which maintained low levels of β-catenin in the cytoplasm (24). GSK-3β has four different phosphorylation regions, and the phosphorylation of the regulatory serine residue 9 in GSK-3β is associated with the inhibition of its kinase activity (25). The present results demonstrated that knockdown of CD147 expression inhibited the phosphorylation of GSK-3β on Ser 9. To confirm that the Wnt/GSK-3β/β-catenin pathway was involved in CD147-induced promotion of EMT in LNCaP cells, LiCl, an activator of the Wnt signalling pathway, was used. LiCl treatment attenuated CD147 knockdown-induced p-β-catenin (Ser33/37/Thr41) expression, which resulted in the upregulation of β-catenin in the nucleus. The expression of Snail and vimentin, as β-catenin targets, was increased by LiCl treatment in LNCaP/shCD147 cells.

In summary, the present study described a novel role of CD147 in the migration of PCa cells. These results demonstrated that CD147 promoted the migration and invasion of PCa cells by suppressing EMT. In addition, CD147 affected the expression of proteins involved in EMT regulation via the Wnt/β-catenin signalling pathway.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LW conceived and designed the study. FF and QI contributed to data acquisition and analysis and drafted the manuscript. MW, CN and HX were involved in data acquisition. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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