CD147 modulates androgen receptor activity through the Akt/Gsk-3β/β-catenin/AR pathway in prostate cancer cells

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Received December 12, 2015; Accepted March 1, 2016

DOI: 10.3892/ol.2016.4684

Abstract. The androgen signaling pathway serves an important role in the development of prostate cancer. β-Catenin is an androgen receptor (AR) cofactor and augments AR signaling. Glycogen synthase kinase-3β (GSK-3β), a target of phosphorylated serine/threonine protein kinase B (p-Akt), regulates β-catenin stability. In addition, β-catenin, a coregulator of AR, physically interacts with AR and enhances AR-mediated target gene transcription. The multifunctional glycoprotein cluster of differentiation (CD) 147 is highly expressed on the cell surface of the majority of cancer cells, and it promotes tumor invasion, metastasis and growth. In the present study, the molecular effects of CD147 on the Akt/GSK-3β/β-catenin/AR signaling network were investigated in LNCaP cells. Using short hairpin-mediated RNA knockdown of CD147 in LNCaP cells, it was demonstrated that downregulation of CD147 resulted in inhibitory phosphorylation of GSK-3β, and then promoted degeneration of β-catenin and reduced nuclear accumulation of β-catenin. In addition, immunoprecipitation studies demonstrated that CD147 downregulation decreased the formation of a complex between β-catenin and AR. It was shown that CD147 knockdown suppressed the expression of the AR target gene prostate-specific antigen and the growth of AR-positive LNCaP cells. Furthermore, inhibition of PI3K/Akt with LY294002 augmented CD147-mediated function. The present study indicates that the PI3K/Akt pathway may facilitate CD147-mediated activation of the AR pathway.

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Key words: CD147, prostate cancer, Akt, GSK-3β, β-catenin, androgen receptor, prostate-specific antigen, proliferation

Introduction

Prostate cancer is one of the leading causes of cancer-associated mortality in men. Prostate cancer cell growth is dependent on the presence of androgens. With an estimated incidence of 233,000 novel cases and 29,480 mortalities in 2014, it is the most frequently diagnosed cancer and second most frequent cause of cancer mortality in American males (1).Although androgen deprivation therapy (ADT) is the standard treatment for metastatic prostate cancer, the tumors tend to relapse following a disease-free period and exhibit androgen-independent proliferation (2), which is termed castration-resistant prostate cancer (CRPC). However, the mechanism underlying CRPC development remains unclear. Previous studies into CRPC have focused on the androgen receptor (AR), including AR amplification and overexpression (3), AR gene mutations (4,5) and the functions of AR co-regulators (6,7). Specifically, AR activity has been shown to be activated by co-regulators at low androgen levels (8), suggesting a critical role of AR co-regulators in prostate cancer progression.

Wang *et al* (7) found evidence of a relationship between the Wnt/β-catenin and androgen signaling pathways (7). β-Catenin, an AR co-regulator, physically interacts with AR through the ligand-binding domain of AR and the first 6 armadillo repeats of β-catenin (9). In prostate cancer cells, β-catenin nuclear localization results in an increased number of AR/β-catenin complexes, thus altering target gene activation. β-catenin activity is regulated by phosphorylation, which leads to its degradation, resulting in decreased nuclear accumulation and decreased interaction with AR. Glycogen synthase kinase-3β (GSK-3β) promotes the phosphorylation and subsequent degradation of β-catenin via the ubiquitin pathway (10).

CD147, also known as extracellular matrix metalloproteinase inducer (EMMPRIN), is highly expressed on the surface of the majority of cancer cells (11). During tumorigenesis, CD147 contributes to metastasis, drug resistance and angiogenesis (12-14). Our previous studies have demonstrated that CD147 is important in prostate cancer cell

invasion, metastasis and autophagy (12,15). In addition, CD147 regulates the canonical Wnt/β-catenin signaling pathway to accelerate lung tumorigenesis (16). The present study aimed to determine whether CD147 mediates the interaction between β-catenin and AR during prostate cancer progression.

Materials and methods

Cell culture and stable transfection of LNCaP cells. The human prostate LNCaP cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). LNCaP cells were maintained in RPMI 1640 medium (Gibco, ThermoFisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal calf serum (FCS; ThermoFisher Scientific, Inc.) at 37˚C in an atmosphere of 95% air and 5% CO_2. To investigate the role of the Akt/GSK-3β pathway in the regulation of β-catenin by CD147, cells were cultured with or without 20 μM LY294002 (Cell Signaling Technology; Danvers, MA, USA) for 24 h. The pGV248 lentiviral vector was used as the backbone for the CD147 shRNA construct. Lentiviral constructs and high-titer viruses were provided by Jikai Genechem Company (Jikai Genechem, Shanghai, China). Cells that expressed low levels of CD147 were termed LNCaP/shCD147 cells. LNCaP/Scramble cells (negative control) were established by transfection of LNCaP cells with the pGV112 vector containing a control shRNA sequence. The target sequences for the CD147 and control shRNA duplexes were 5'-GTCGTCAGAACACATCAACT-3' and CAGTCG CGTTTGCGACTGG, respectively. Lentiviral infection was performed following the manufacturer's protocol. Briefly, $1x10^6$ cells were seeded per well in 12-well plates 16-18 h prior to the experiment. Recombinant lentiviral vectors (10 μg) were produced by co-transfecting 293T cells with the lentivirus expression plasmid (10 μg) and packaging plasmid (10 μg, pHelper 1.0 and pHelper 2.0) using Lipofectamine 2000 (Invitrogen, Shanghai, China). After 48 h, the supernatants were collected and concentrated.

Western blotting. Cells were washed twice with PBS and then lysed with RIPA lysis buffer (Beyotime, Inc., Nanjin, China) containing a cocktail of protease inhibitors (Roche Diagnostics, Mannheim, Germany). To extract specific protein compartments, the Compartmental Protein Extraction Kit (Millipore, Billerica, MA, USA) was used according to the manufacturer's protocol. Cell lysates containing 30 μg of protein were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies against β-catenin (cat no. 8480), p-β-catenin (Ser 33/37/Thr 41; cat no. 9561), p-Akt (Ser 473; cat no. 4060), p-GSK-3β (Ser 9; cat no. 9322) and prostate-specific antigen (PSA; cat no. 5365) (1:1,000; Cell Signaling Technology; Danvers, MA, USA) overnight at 4˚C. β-Actin was used as a loading control (1:2000; Epitomics, Burlingame, CA, USA). Primary antibody binding was detected using a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:3,000; cat no. A0208; Beyotime Institute of Biotechnology) and was visualized using an enhanced chemiluminescence (ECL) detection system (Pierce, ThermoFisher Scientific, Inc., Rockford, IL, USA).

Immunoprecipitation. Cells were lysed using lysis buffer containing 20 mM Tris-HCl (pH 7.8), 0.5% Nonidet P-40, 137 mM NaCl, 50 μM EDTA, and protease inhibitors (Roche Diagnostics, Mannheim, Germany), and protein extracts were incubated with 2 μg anti-AR mouse IgG antibody (cat no. sc-31358; Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4˚C, followed by the addition of protein A/G agarose beads (Santa Cruz Biotechnology) for 2 h. After 3 washes with 0.5 ml of lysis buffer, the pellets were suspended in SDS sample buffer, boiled for 5 min, and analyzed on 10% SDS-polyacrylamide gels. Proteins were transferred to PVDF membranes (Millipore, Billerica, MA, USA), and western blot analysis was performed with a rabbit anti-β-catenin antibody (1:1,000; Cell Signaling Technology, Danvers, MA, USA). Primary antibody binding was detected using a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:3,000; cat no. A0208; Beyotime Institute of Biotechnology) and was visualized using an enhanced chemiluminescence (ECL) detection system (Pierce, ThermoFisher Scientific, Inc., Rockford, IL, USA).Control immunoprecipitation experiments were performed with normal immune serum (Beyotime, Inc., Nanjin, Jiangsu, China).

Cell growth assays. Cell growth was determined using the Cell Counting Kit-8 (CCK-8, Dojindo, Japan). Briefly, cells were seeded in 96-well plates at a density of $3x10^3$ cells/well and cultured for 4, 8 and 12 days. CCK-8 solution was added to each well, and cells were incubated for an additional 3 h. Each experiment was performed in triplicate and repeated 3 times. The absorbance at 450 nm was measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Inhibition rate (%)=$(A_{450,\text{ control group}}-A_{450,\text{ experimental group}})/A_{450,\text{ control group}}x100$.

Statistical analysis. The data are presented as the mean ± standard deviation. One-way analysis of variance (ANOVA) was used to compare significant differences in the means among all treatment groups, and Bonferroni's correction was used to identify significant statistical differences between the means of individual treatments. $P<0.05$ was considered statistically significant. All statistical analyses were performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA).

Results

CD147 regulates β-catenin nuclear localization. β-Catenin is a multifunctional protein that has structural importance in the adhesive junction complex and the ability to bind to AR to stimulate AR-mediated gene transcription (17). β-catenin expression was measured in LNCaP/shCD147 and LNCaP/Scramble cells by western blotting. LNCaP/shCD147 cells exhibited significantly lower β-catenin protein levels in the cytoplasm ($P<0.01$) and nucleus ($P<0.01$) compared with LNCaP/Scramble cells (Fig. 1). These results indicate that CD147 is a key molecule involved in β-catenin localization.

CD147 regulates β-catenin stability via the Akt/GSK-3β signaling pathway in LNCaP cells and affects AR/β-catenin protein-protein interactions. β-Catenin stability is triggered by GSK-3β, a well-known target of Akt; therefore, the present

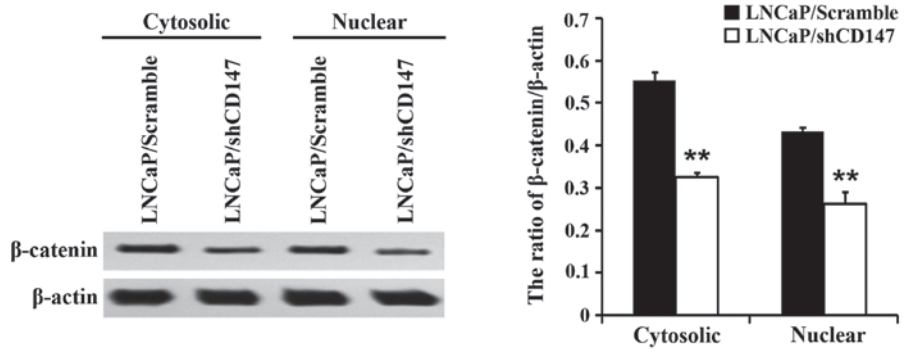

Figure 1. CD147 induces β-catenin nuclear localization. LNCaP cells were transfected with CD147 shRNA-encoding lentivirus or control lentivirus. Cytoplasmic and nuclear lysates from each sample were subjected to western blot analysis. β-Actin was used as an internal control. **P<0.01 vs. LNCaP/Scramble cells. The results are representative of 3 separate experiments with similar results.

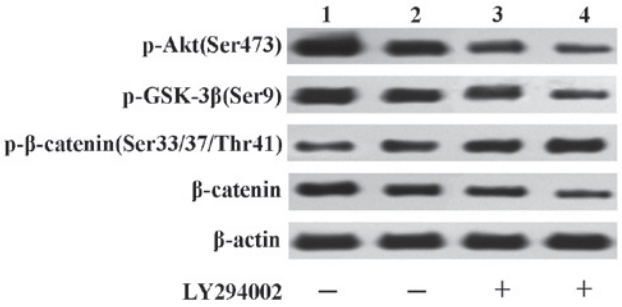

Figure 2. The Akt/GSK-3β signaling pathway mediates β-catenin stability via CD147. LNCaP/shCD147 and LNCaP/Scramble cells were pre-treated with or without LY294002 (20 μM), and the expression levels of p-Akt (Ser473), p-GSK-3 (Ser9), p-β-catenin (Ser33/37/Thr41) and β-catenin were examined by western blot analysis. β-Actin was used as an internal control. Lanes 1 and 3, LNCaP/Scramble group; Lanes 2 and 4, LNCaP/shCD147 group. The results are representative of 3 separate experiments with similar results.

study focused on Akt/GSK-3β signaling. GSK-3β activity is suppressed by phosphorylation on Ser 9 by Akt. To investigate the role of the Akt/GSK-3β pathway in the regulation of β-catenin by CD147, the expression of p-Akt (Ser473), p-GSK-3β (Ser9), p-β-catenin (Ser33/37/Thr41) and β-catenin was examined in LNCaP/shCD147 and LNCaP/Scramble cells cultured with or without 20 μM LY294002, a specific inhibitor of class I phosphatidylinositol-3-kinases (PI3Ks). Expression of p-Akt (Ser473) and p-GSK-3β (Ser9) were down-regulated in LNCaP/shCD147 cells, as expected, which was consistent with the down-regulation of β-catenin and the up-regulation of p-β-catenin (Ser33/37/Thr41) (Fig. 2). In support of these findings, exposure to LY294002 significantly decreased p-Akt (Ser473), p-GSK-3β (Ser9) and β-catenin levels and increased p-β-catenin (Ser33/37/Thr41) levels in LNCaP/shCD147 cells (Fig. 2). β-Catenin binds to AR and stimulates AR-mediated gene transcription. Protein extracts from LNCaP/Scramble and LNCaP/shCD147 cells were immunoprecipitated with an anti-AR antibody, followed by SDS-PAGE and western blotting analysis using an anti-β-catenin antibody with normal IgG as a negative control. Fig. 3 demonstrates that introducing CD147-shRNA into LNCaP cells reduced the formation of the AR/β-catenin complex. As expected, AR/β-catenin complex formation was lowest when LNCaP/shCD147 cells were treated with LY294002 (Fig. 3; P<0..01). These results suggest that CD147 enhances the stability of β-catenin via the Akt/GSK-3β pathway, which leads to increased AR/β-catenin protein-protein interactions.

CD147 promotes AR-mediated gene expression. PSA is a well-established AR target gene in human prostate cancer. Therefore, the potential effect of CD147 on AR-mediated PSA protein expression was investigated using western blot analysis. As shown in Fig. 4, shRNA targeting CD147 resulted in reduced PSA protein levels (P<0.05). In addition, LNCaP cells were treated with LY294002 and PSA protein levels were further reduced in LNCaP/shCD147 cells (Fig. 4; P<0.05). Consistent with the above results, CD147 appears to enhance the expression of downstream AR target proteins via the Akt/GSK-3β/β-catenin pathway.

CD147 enhances the growth of prostate cancer cells. The role of CD147 in the regulation of prostate cancer cell growth was also investigated because LNCaP cells are androgen-sensitive and AR-positive. Cell growth was measured using the CCK8 assay. As shown in Fig. 5, knocking down CD147 expression in LNCaP cells inhibited cell growth compared with control Scramble expression (P<0.01). Moreover, the growth inhibition rate in the LNCaP/shCD147 group was markedly increased by LY294002 treatment on days 4, 8 and 12 compared with the control LNCaP/shCD147 group (P<0.01).

Discussion

The AR signaling pathway has been demonstrated to be involved in CRPC. Emerging evidence indicates that β-catenin is more highly expressed in prostate cancer than in normal prostate tissue and that it is involved in advanced prostate cancer (18). In prostate cells, β-catenin directly interacts with AR and acts as a co-activator to enhance androgen-induced AR-mediated transcription. A previous study demonstrated that the dysregulation of β-catenin and the androgen-signaling pathway induces cell growth and contributes to the initiation and progression of prostate cancer (19).

CD147 serves an important role in tumor biology; it inhibits cancer cell anoikis and promotes cancer cell invasion and metastasis. In the present study, a previously unknown effect of CD147 on the β-catenin signaling pathway in prostate cancer

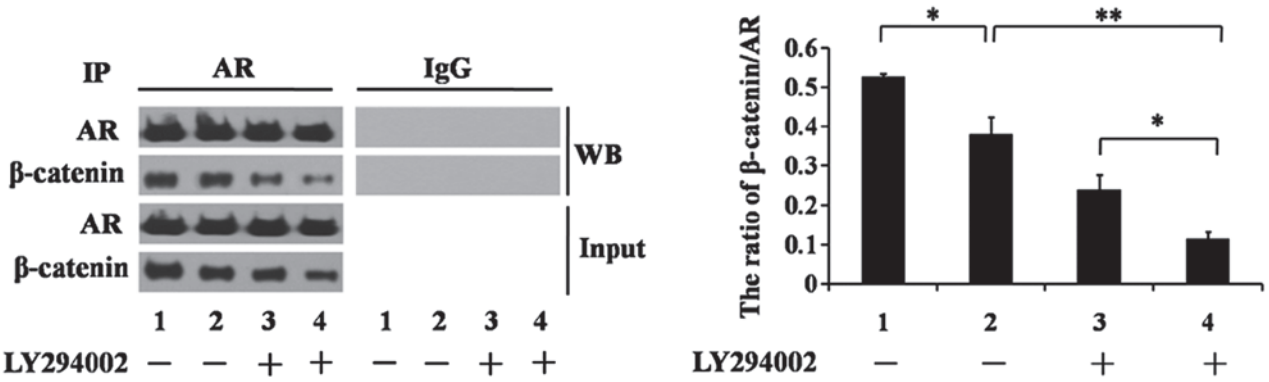

Figure 3. CD147 enhances the interaction between AR and β catenin. LNCaP/shCD147 and LNCaP/Scramble cells were incubated with or without LY294002 (20 μM), and whole cell extracts were prepared. Immunoprecipitation experiments were performed using an anti AR antibody, and the precipitates were subjected to immunoblotting with an anti β-catenin antibody. LNCaP/Scramble cell lysates were also run directly on the gel to demonstrate the presence of β-catenin and AR. Lanes 1 and 3, LNCaP/Scramble group; Lanes 2 and 4, LNCaP/shCD147 group. The results are representative of 3 separate experiments with similar results. AR, androgen receptor; WB, western blot; IP, immunoprecipitation. *P<0.05, **P<0.01.

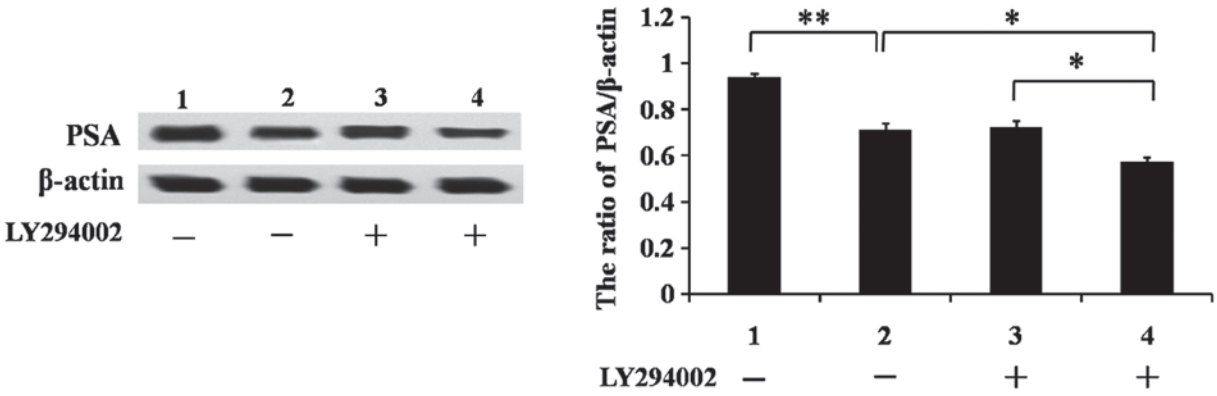

Figure 4. CD147 promotes AR mediated PSA expression. LNCaP/shCD147 and LNCaP/Scramble cells were incubated with or without LY294002 (20 μM), and whole cell extracts were prepared. Protein levels were detected by western blotting with an anti PSA antibody. β-actin was used as an internal control. Lanes 1 and 3, LNCaP/Scramble group; Lanes 2 and 4, LNCaP/shCD147 group. The results are representative of 3 separate experiments with similar results. AR, androgen receptor; PSA, prostate specific antigen. *P<0.05, **P<0.01.

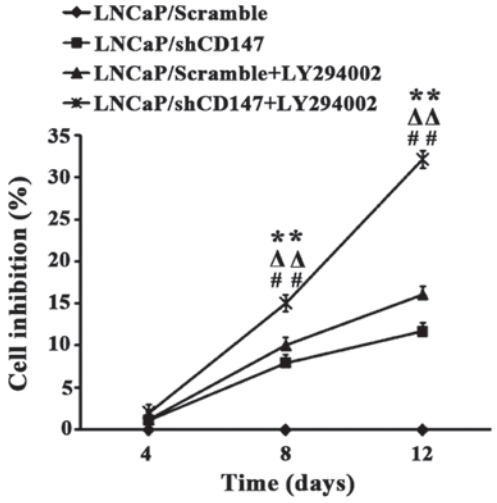

Figure 5. CD147 augments prostate cancer cell proliferation. LNCaP/shCD147 and LNCaP/Scramble cells were seeded in 96-well plates and incubated for 4, 8 or 12 days with or without LY294002 (20 μM). Cell proliferation was determined using a cell counting kit-8 assay. Values are presented as the mean ± standard error of 3 experiments. **LNCaP/shCD147 P<0.01 vs. LNCaP/Scramble; $^{\Delta\Delta}$LNCaP/shCD147+LY294002 P<0.01 vs. LNCaP/shCD147; $^{\#\#}$LNCaP/shCD147+LY294002 P<0.01 vs LNCaP/Scramble+LY294002.

was identified. It was demonstrated that knockdown of CD147 expression decreased β-catenin levels and disrupted the nuclear accumulation of active β-catenin in LNCaP cells. Decreased β-catenin level may result from the GSK-3β-mediated phosphorylation of β-catenin on Ser 33/37 and Thr 41. The phosphorylated form of β-catenin is ubiquitinated by the β-TrCP ubiquitin E3 ligase degraded by the proteasome (20). The data in the present study demonstrated that CD147 knockdown inhibited the phosphorylation of GSK-3β on Ser 9 and increased the phosphorylation of β-catenin on Ser 33/37 and Thr 41, which can induce β-catenin ubiquitination. The results demonstrated that knockdown of CD147 expression could potentially lead to an increase GSK-3β-mediated phosphorylation of β-catenin. Phosphorylation of β-catenin leads to its instability and decreases its accumulation in the nucleus. AR-mediated target gene transcription is essential for prostate cancer growth and progression (21). β-catenin acts as an AR co-activator and enhances AR-mediated gene transcription (9). PSA, a critical downstream AR target gene, is an important biomarker of disease onset and progression in prostate cancer. In the present study, it was observed that the interaction between β-catenin and AR was decreased, ultimately leading

to the inhibition of PSA expression in LNCaP/shCD147 cells compared with LNCaP/Scramble cells. A previous study showed that dysregulation of the Wnt and androgen signaling pathways induces cell growth and directly contributes to the progression of prostate cancer (19). In the present study, the data indicated that decreased CD147 expression inhibited AR-positive LNCaP cell growth, which is consistent with the role of CD147 in enhancing AR-mediated transcription. These results suggest that CD147 contributes to novel molecular cross-talk between Wnt/β-catenin signaling and AR activity.

The class I PI3K/Akt pathway regulates a wide spectrum of cellular processes, including cell cycle progression, cell survival and migration (18). Dysregulation of the PI3K/Akt pathway promotes tumorigenesis and angiogenesis in various cancer types (18). Our prior study demonstrated that CD147 activates the class I PI3K/Akt pathway in prostate cells (15). GSK-3β also serves an important role in suppressing tumor progression through the cytoplasmic phosphorylation and degradation of β-catenin via the Akt signaling pathway (22). Therefore, the present authors hypothesized that the PI3K/Akt pathway may be involved in the regulation of β-catenin by CD147. It was demonstrated that decreasing CD147 levels inhibited the Akt phosphorylation and significantly decreased GSK-3β activity through N-terminal phosphorylation at Ser 9 and that LY294002, a specific inhibitor of class I PI3Ks, further destabilized β-catenin. Therefore, PI3K/Akt signaling is potentially involved in CD147-mediated β-catenin stabilization, resulting in significant inhibition of the interaction between β-catenin and AR and decreased PSA levels.

The current study prevents evidence that CD147 acts as an upstream signal that modulates Akt/GSK-3β/β-catenin/AR signaling network and promote prostate cell proliferation. These results provide novel insights into the function of CD147 during tumor progression.

Acknowledgements

The present study was supported by grants from the China National Natural Science Foundation (grant no. 81202031) and the Science and Technology Development Program of Jilin Province (grant no. 20130101154JC).

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