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Overexpression of Glypican 5 (GPC5) Inhibits Prostate Cancer Cell Proliferation and Invasion via Suppressing Sp1-Mediated EMT and Activation of Wnt/β-Catenin Signaling

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Glypican 5 (GPC5) belongs to the family of heparan sulfate proteoglycans (HSPGs). It was initially known as a regulator of growth factors and morphogens. Recently, there have been reports on its correlation with the tumorigenic process in the development of some cancers. However, little is known about its precise role in prostate cancer (PCa). In the present study, we explored the expression pattern and biological functions of GPC5 in PCa cells. Our results showed that GPC5 was lowly expressed in PCa cell lines. Upregulation of GPC5 significantly inhibited PCa cell proliferation and invasion in vitro as well as attenuated tumor growth in vivo. We also found that overexpression of GPC5 inhibited the epithelial–mesenchymal transition (EMT) and Wnt/β-catenin signaling activation, which was mediated by Sp1. Taken together, we suggest GPC5 as a tumor suppressor in PCa and provide promising therapeutic strategies for PCa.

Key words: Glypican 5 (GPC5); Prostate cancer (PCa); Proliferation; Invasion; Epithelial–mesenchymal transition (EMT)

INTRODUCTION

Prostate cancer (PCa), a common malignant tumor in males, originates from epithelial tissues like many other types of cancers[1,2]. At the early stage, PCa features slow growth and low invasiveness[3,4]. For patients at this stage, tumors are confined to the prostate gland and cause only limited symptoms[5]. Therefore, patients frequently have good prognosis after specific treatment. However, PCa becomes more invasive at the late stage, and previous therapies lose their effectiveness, which frequently results in the death of patients[6–8]. Thus, it is of great importance to identify invasiveness-related markers and elucidate the molecular mechanisms underlying the progression of PCa.

Glypicans (GPCs), a group of proteoglycans, belong to the family of heparan sulfate proteoglycans (HSPGs)[9]. So far, there have been six members identified in this group, which are GPC1 to GPC6[10]. These proteoglycans, located in the extracellular matrix, are anchored, via glycosyl-phosphatidylinositol, to the external surface of plasma membranes[11]. Initially, GPCs were known as the regulators of growth factors and morphogens[12]. Recently, some GPCs have been reported to be correlated with the tumorigenic process in the development of various cancers. For example, GPC3 has been found to show an increased expression level in hepatocellular carcinoma (HCC) and exert a promoting effect on HCC cell growth[13,14]. GPC5, exhibiting a high homology to GPC3, has also been investigated by many researchers for its potential as a tumor suppressor in lung and breast cancers[15–17]. However, to the best of our knowledge, there have been no investigations on the biological functions of GPC5 in PCa.

In the present study, we found that GPC5 was lowly expressed in PCa cell lines and that upregulation of GPC5 inhibited PCa cell proliferation and invasion in vitro as well as attenuated tumor growth in vivo. The results also showed that GPC5-inhibited epithelial–mesenchymal transition (EMT) and Wnt/β-catenin activation were mediated by specificity protein 1 (Sp1). These novel findings suggest GPC5 as a tumor suppressor in PCa and provide promising therapeutic strategies for PCa.

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MATERIALS AND METHODS

Cell Lines and Cell Culture

Human PCa cell lines (DU-145 and PC-3) and a normal prostatic epithelial cell line (RWPE-1) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/ml penicillin (Gibco), and 100 mg/ml streptomycin (Gibco), at 37°C in a humidified incubator with 5% CO_2.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

After extraction from cells by TRIzol (Invitrogen) reagent, RNA was reversely transcribed into cDNA using Moloney murine leukemia virus (MMLV) reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). RT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) under the following conditions: 95°C for 10 min, 95°C for 5 s, 50 cycles of 60°C for 30 s, and 72°C for 5 min. The primers were as follows: GPC5, 5′-CCCTCGAGGGA GGATGGACGCACAGACC-3′ (forward) and 5′-CGG GATCCCGCCAGGCATATGCAGAGAGAGAG-3′ (reverse); GAPDH, 5′-CAATGACCCCTTCATTGACC-3′ (forward) and 5′-TGGAAGATGGTGATGGGATT-3′ (reverse). GAPDH was used as an internal control. The relative expression of genes was calculated using the $2^{-\Delta\Delta}$Ct method.

Western Blot

Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (Invitrogen). After centrifugation of cell lysates, the supernatant was collected and then quantified using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Protein was separated by 12% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequent to blocking with 5% nonfat milk, the membranes were incubated overnight at 4°C with primary antibodies against GPC5, E-cadherin, α-catenin, N-cadherin, vimentin, β-catenin, c-Myc, and cyclin D1 and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by washing three times with TBST buffer and exposure to appropriate secondary antibodies (Santa Cruz Biotechnology). The protein was visualized using ECL detection reagents (Thermo Fisher Scientific) and quantified via the Tanon GIS system (Tanon, Shanghai, P.R. China).

Lentivirus Infection

Cells were infected with a lentivirus containing the pLV-GPC5-GFP or pLV-GFP vector. Subsequent to cotransfection of the target plasmid, the virus was packaged in 293-T cells. Condition medium was applied for virus titration 48 h after transfection. Cells were infected with the filtered lentivirus and polybrene (2 mg/ml; Sigma-Aldrich, St. Louis, MO, USA). After growing for 48 h in complete medium with 10% fetal calf serum (FCS) and 400 μg/ml of G418 (Gibco), cells were selected and isolated.

MTT Assay

Cell proliferation was measured by the MTT assay. Briefly, cells were seeded in 96-well plates at a density of 2×10^4 cells/well and cultured for 24, 48, 72, or 96 h, respectively. Then 20 ml of MTT reagent (5 mg/ml; Sigma-Aldrich) was added to each well at different time points, and the cells were further incubated for 4 h. After removal of the supernatant, dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to each well. The OD value was measured with a microplate reader (Bio-Rad) at a wavelength of 570 nm.

Transwell Assay

Transwell chambers with a Matrigel-coated polycarbonate membrane (BD Biosciences, Franklin Lakes, NJ, USA) were used to evaluate cell invasion. Cells (5×10^4) were suspended in serum-free medium (150 μl) and added to the upper chamber. Culture medium with 10% FBS was added to the lower chamber. After 24 h of incubation, cells on the upper surface of the membrane were removed with a cotton swab, and cells on the lower surface of the membrane were fixed and stained with 0.1% crystal violet. The invading cells from five random fields were counted under a microscope at 200× magnification.

In Vivo Xenograft Tumor Assay

Male BALB/c nude mice (4 to 6 weeks) were obtained from the Shanghai Laboratory Animal Center (Shanghai, P.R. China). All animal experiments were performed according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Ethics Committee of the General Hospital of Daqing Oil Field (Daqing, P.R. China). PC-3 cells (5×10^6) transfected with pCMV-Taq2B-GPC5 or pCMV-Taq2B were resuspended in 0.1 ml of PBS and injected subcutaneously into the left flank of nude mice ($n=8$). The tumor size was measured every week and was calculated according to the following formula: $(\text{length}\times\text{width}^2)\times1/2$. Five weeks later, the mice were sacrificed, and the tumors were stripped and weighed.

Statistical Analysis

The results were expressed as means ± standard deviation (SD). The comparison of the experimental data

was made via Student's *t*-tests. SPSS 13.0 software was used for statistical analysis. A value of $p<0.05$ was considered statistically significant.

RESULTS

GPC5 Is Lowly Expressed in PCa Cell Lines

To investigate the role of GPC5 in PCa, we first detected its expression levels in PCa cell lines (DU-145 and PC-3) and a normal prostatic epithelial cell line (RWPE-1) by RT-PCR and Western blot analysis. We found that both the mRNA and protein levels of GPC5 in the DU-145 and PC-3 cell lines were significantly reduced in comparison with the RWPE-1 cell line (Fig. 1A and B).

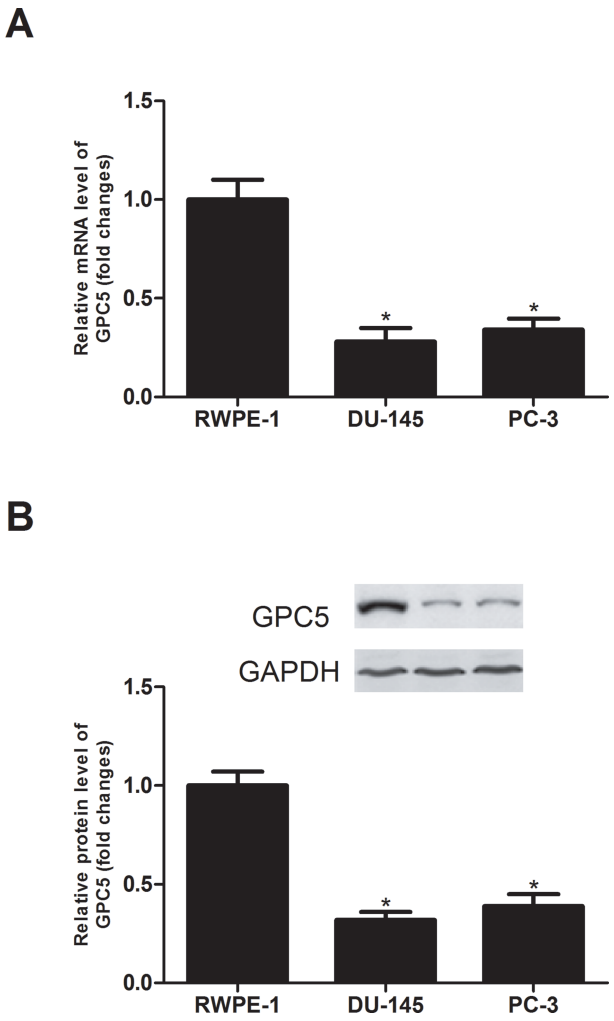

Figure 1. Glypican 5 (GPC5) is lowly expressed in prostate cancer (PCa) cell lines. (A) The mRNA expression levels of GPC5 in PCa cell lines were determined by real-time polymerase chain reaction (RT-PCR) ($n=30$). (B) The protein levels of GPC5 in PCa cell lines were determined by Western blot analysis. *$p<0.05$.

Overexpression of GPC5 Inhibits PCa Cell Proliferation and Invasion In Vitro as well as Attenuates Tumor Growth In Vivo

To determine the effect of GPC5 on PCa cells, we increased its expression by infection of PC-3 cells with the pLV-GPC5-GFP-containing lentivirus. RT-PCR and Western blot analysis were performed to confirm overexpression of GPC5 in PC-3 cells (Fig. 2A and B).

The MTT assay was used to evaluate the impact of GPC5 on PCa cell proliferation. The results showed that the proliferative ability of PC-3 cells was greatly inhibited after the increase in the expression of GPC5 (Fig. 2C). The Transwell assay was performed to test cell invasion. As shown in Figure 2D, overexpression of GPC5 remarkably decreased the number of invading PC-3 cells, in comparison with the control cells. Xenograft tumor models were established to further examine the role of GPC5 in the regulation of PCa tumor growth in vivo. As shown in Figure 2E and F, the tumor volume and weight were significantly reduced in tumors derived from GPC5-transfected cells in comparison with those from the control cells.

Overexpression of GPC5 Inhibits the EMT Process in PCa Cells

EMT is a crucial mechanism leading to cancer cell invasion[18]. Therefore, we investigated whether GPC5 inhibited PCa cell invasion by reversing EMT. For this purpose, we measured the expression of the epithelial markers (E-cadherin and α-catenin) and the mesenchymal markers (N-cadherin and vimentin) in PC-3 cells. The results showed that overexpression of GPC5 increased the protein expression of E-cadherin and α-catenin but decreased that of N-cadherin and vimentin in PC-3 cells (Fig. 3A and B).

Overexpression of GPC5 Inhibits the Activity of Wnt/β-Catenin Signaling

The Wnt/β-catenin pathway is an essential player in the progression of various cancers including PCa[19]. β-Catenin, an important component of the Wnt signaling pathway, is a critical regulator of cell proliferation and invasion[20,21]. Thus, we measured the effect of GPC5 on the expression of β-catenin and its well-known target genes c-Myc and cyclin D1. As shown in Figure 4A and B, the protein levels of β-catenin, c-Myc, and cyclin D1 were downregulated by GPC5 overexpression in PC-3 cells, indicating an inhibitory effect of GPC5 on the activity of Wnt/β-catenin signaling.

GPC5-Inhibited EMT and Wnt/β-Catenin Activation Is Mediated by Sp1

We considered Sp1 as a key mediator of GPC5-inhibited EMT and Wnt/β-catenin activation because of

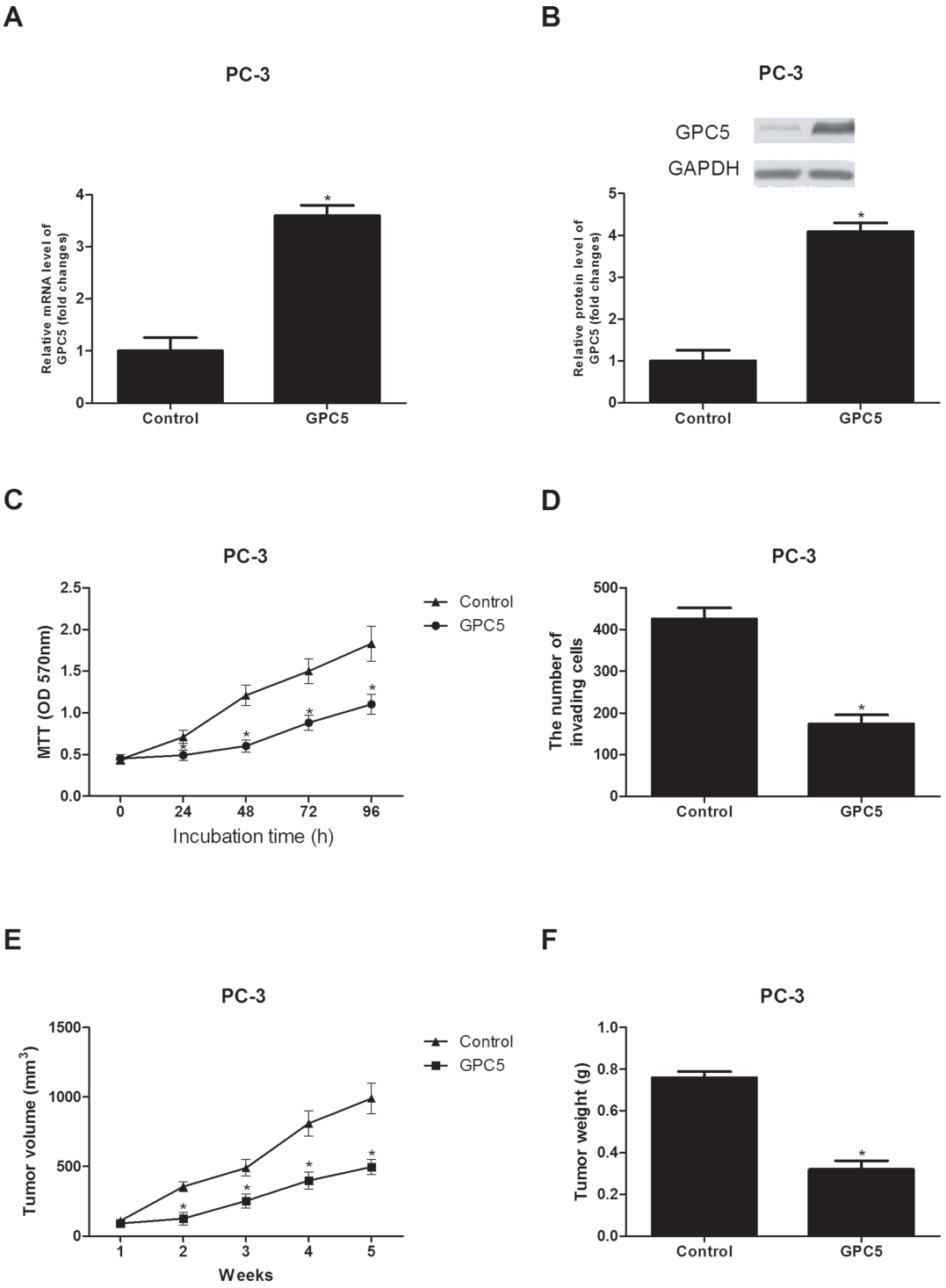

Figure 2. Overexpression of GPC5 inhibits PCa cell proliferation and invasion in vitro as well as attenuates tumor growth in vivo. (A, B) Relative mRNA and protein levels of GPC5 in PC-3 cells were markedly increased by infection of the pLV-GPC5-GFP-containing lentivirus, in comparison with the control cells. (C, D) The proliferative and invasive capabilities of PC-3 cells were greatly reduced by GPC5 overexpression. (E) The tumor volume was measured every week after injection. (F) Tumors were weighed 5 weeks after injection ($n=8$). *$p<0.05$.

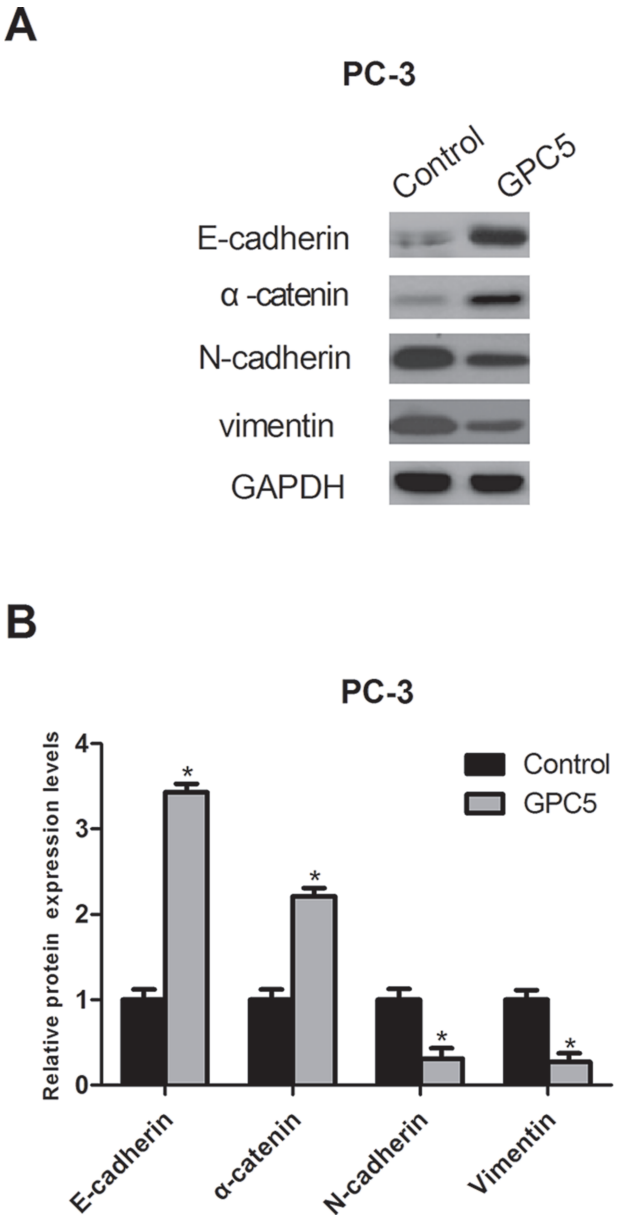

Figure 3. Overexpression of GPC5 inhibits the epithelial–mesenchymal transition (EMT) process in PCa cells. (A) The protein expression of EMT-related factors was detected in PC-3 cells using Western blot analysis after GPC5 overexpression. (B) The protein expression levels of EMT-related factors were quantified via the Tanon GIS system. *$p<0.05$.

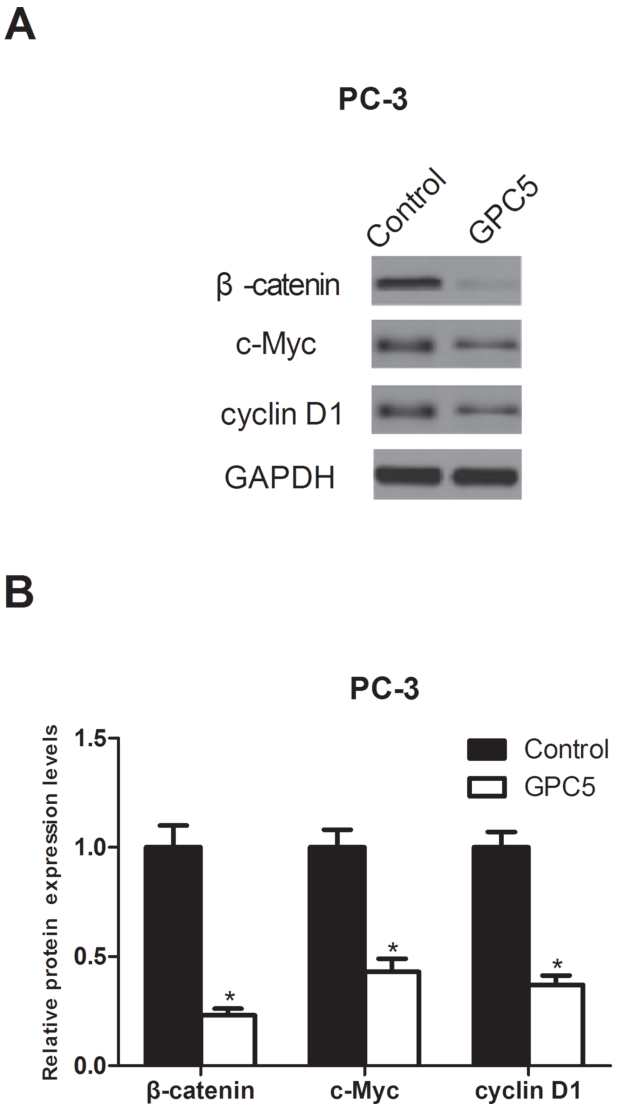

Figure 4. Overexpression of GPC5 inhibits the activity of Wnt/β-catenin signaling. (A) The protein expression of β-catenin, c-Myc, and cyclin D1 was detected in PC-3 cells after GPC5 overexpression. (B) The protein expression of β-catenin, c-Myc, and cyclin D1 was quantified via the Tanon GIS system. *$p<0.05$.

its potential role as a transcription factor implicated in cell proliferation and invasion as well as its involvement in the EMT process of some types of cancers[22,23]. To test our hypothesis, we measured the expression of Sp1 after GPC5 overexpression. As expected, the protein levels of Sp1 expression were reduced in GPC5-transfected PC-3 cells in comparison with the control cells (Fig. 5A). To ascertain the necessity of Sp1 for GPC5-inhibited effects, we detected the expression of β-catenin, c-Myc, cyclin D1, and vimentin in PC-3 cells treated with mithramycin A (100 nM), an inhibitor of Sp1. The results showed that mithramycin A potentiated the inhibitory effect of GPC5 on the protein levels of β-catenin, c-Myc, cyclin D1, and vimentin (Fig. 5B).

DISCUSSION

In recent years, PCa has shown a gradual trend of high incidence around the world and thus became a great healthcare challenge[24,25]. The main research focus lies in

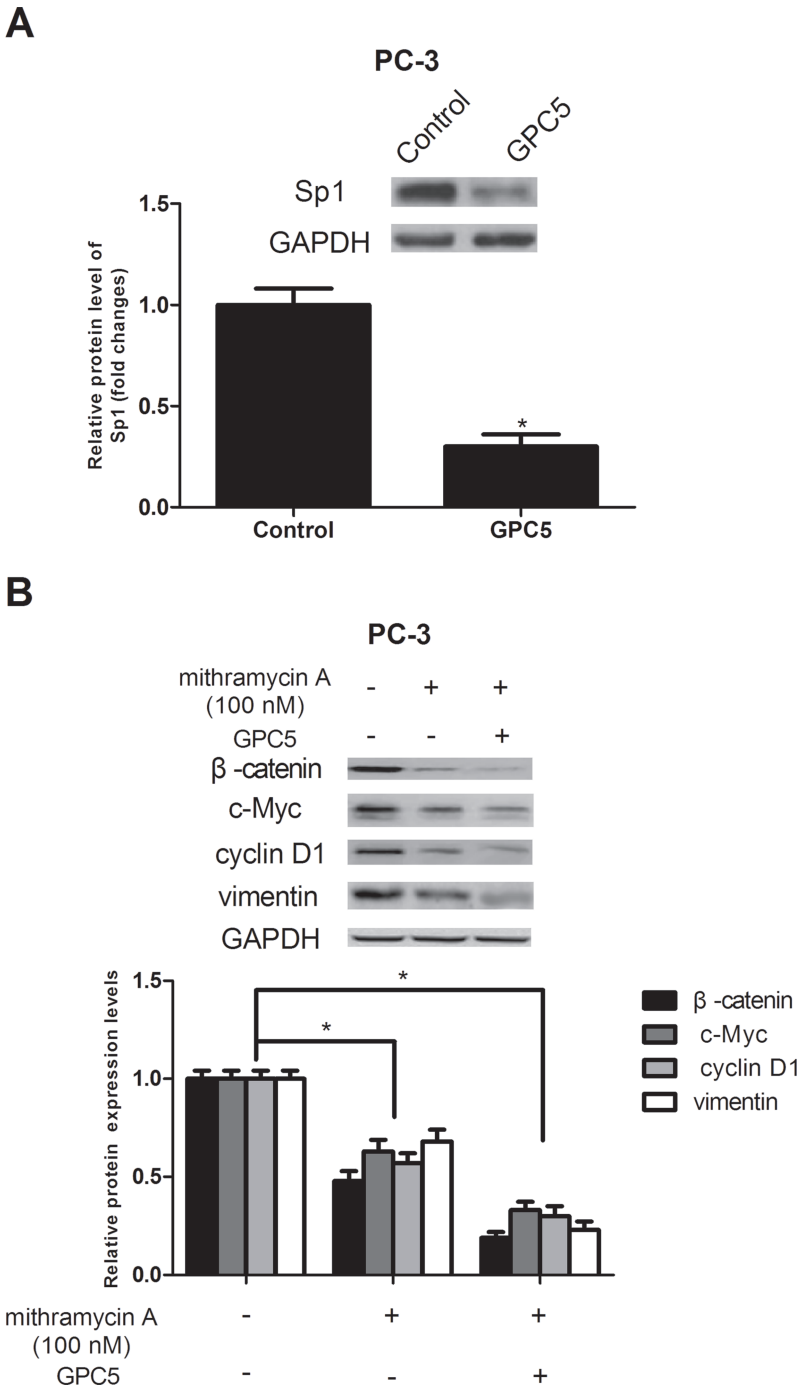

Figure 5. GPC5-inhibited EMT and Wnt/β-catenin activation are mediated by Sp1. (A) The protein expression of Sp1 was detected in PC-3 cells after overexpression of GPC5. (B) The protein expression of β-catenin, c-Myc, cyclin D1, and vimentin was detected in PC-3 cells in the presence or absence of mithramycin A (100 nM). *$p < 0.05$.

the increased cell proliferation and invasion because they are key processes for PCa progression and are widely considered as important hallmarks of PCa[26].

In the present study, we demonstrated that GPC5 was lowly expressed in PCa cell lines and that upregulation of GPC5 inhibited PCa cell proliferation and invasion in vitro as well as attenuated tumor growth in vivo. Based on these results, we considered GPC5 as an essential player in suppressing the development of PCa. As previously mentioned, low expression of GPC5 has been found

in many cancers, suggesting that GPC5 downregulation may be a common characteristic in tumor progression. Moreover, a growing number of studies have indicated the anticancer effect of GPC5. For example, Yuan et al. observed that GPC5 was a novel epigenetically silenced tumor suppressor and could suppress tumor growth of lung cancer[27]. However, in gastric cancer and HCC, GPC5 was reported to function as an oncogene and used as a promising target for cancer treatment[9,28]. This controversial role of GPC5 may be caused by the types of cancer cells. Thus, the biological functions of GPC5 in cancers are required to be further investigated.

EMT, with a positive relation to invasion, is a crucial factor for cancer progression and is featured by the loss of epithelial markers and the gain of mesenchymal markers[29–31]. In this study, we found that overexpression of GPC5 altered the expression of EMT markers, thus inhibiting PCa cell invasion. We also demonstrated that the increase in the expression of GPC5 suppressed the activity of Wnt/β-catenin signaling. The Wnt/β-catenin pathway plays a critical role in cancer progression. β-Catenin, an important regulator of cell proliferation and invasion, is frequently activated in PCa[32–34]. Our results showed that GPC5 overexpression significantly reduced the protein expression of β-catenin and its crucial target genes c-Myc and cyclin D1 in PCa cells. Regarding the mechanisms underlying GPC5-inhibited EMT and Wnt/β-catenin activation, we investigated and found Sp1 to be important in the regulation. As a transcription factor, Sp1 is involved not only in cell proliferation and invasion but also in the EMT process. For example, Zhang et al. reported that the expression levels of Sp1 were upregulated in nasopharyngeal cancer cell lines and that downregulation of Sp1 inhibited nasopharyngeal cancer cell proliferation[22]. Kwon et al. found that Sp1 increased E-cadherin repressors and subsequently induced EMT[26]. In addition, Sp1 has a reciprocal action with Wnt/β-catenin signaling by activating the target genes of the Wnt/β-catenin pathway during cancer development[35,36]. Notably, we showed that GPC5 overexpression decreased the protein levels of Sp1 in PCa cells, and the use of mithramycin A (an inhibitor of Sp1) enhanced the inhibitory effect of GPC5 on the protein levels of β-catenin, c-Myc, cyclin D1, and vimentin.

In conclusion, this study first identified GPC5 as a tumor suppressor in PCa and reported the molecular mechanism underlying GPC5-regulated cancer progression. The results showed that overexpression of GPC5 inhibited PCa cell proliferation and invasion in vitro as well as suppressed tumor growth in vivo. Our study further indicated that Sp1 was essential for GPC5-inhibited EMT and Wnt/β-catenin signaling activation. These novel findings expanded our understanding about the role of GPC5 in PCa progression and provided helpful strategies for PCa treatment.

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