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High PLK4 expression promotes tumor progression and induces epithelial-mesenchymal transition by regulating the Wnt/β-catenin signaling pathway in colorectal cancer

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Abstract. Polo-like kinase 4 (PLK4) has been identified as an oncogene, which is overexpressed in various types of human cancer; however, its role in colorectal cancer (CRC) development remains unknown. The present study demonstrated that PLK4 protein expression was upregulated in CRC tissues compared with in normal tissues through western blotting. In addition, immunohistochemical analysis of 39 CRC specimens further demonstrated that PLK4 protein expression was upregulated in 64.1% (25/39) of samples. Increased PLK4 expression was closely associated with enhanced tumor size (P=0.031), lymph node metastasis (P=0.016) and TNM stage (P=0.001). Subsequently, cell viability, wound scratch, migration and invasion assays were conducted *in vitro*, and nude mice CRC xenograft models were generated. The results demonstrated that knockdown of PLK4 in CRC cells resulted in significant decreases in cell viability and proliferation, and decreased the protein expression levels of N-cadherin and snail, which are biomarkers of epithelial-mesenchymal transition. Furthermore, PLK4 knockdown inactivated the Wnt/β-catenin pathway in CRC cells *in vitro* and *in vivo*, and suppressed the growth of xenograft tumors in nude mice. In conclusion, these results suggested that PLK4 may promote the carcinogenesis and metastasis of CRC, thus indicating that PLK4 may be considered a molecular target for CRC treatment.

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Introduction

Colorectal cancer (CRC) is one of the leading global causes of cancer-associated mortality (1,2). Although improvements have been made to the diagnosis and treatment of CRC, the effects of surgery and chemotherapy are still not satisfactory, and the 5-year survival rate for patients with CRC remains low (3,4). Traditional clinicopathological parameters, including TNM stage and serum carcinoembryonic antigen levels, have been widely used in prognostic evaluation; however, most of these parameters cannot fully predict individual clinical results (5). Therefore, novel diagnosis and treatment methods are required to improve the prognosis of patients with CRC.

Polo-like kinase 4 (PLK4), which is a member of the polo family of serine/threonine protein kinases, localizes to centrioles, which are complex microtubule-based structures present in centrosomes (6,7). PLK4 functions primarily as a regulator that mediates centriole duplication during the cell cycle (8). In addition, the oncogenic role of PLK4 has been studied in various types of cancer (9-15). The expression of PLK4 has been detected in breast cancer (11,14), lung cancer (12), neuroblastoma (13) and prostate cancer (15); however, its expression pattern differs among various types of cancer. Increased PLK4 expression is correlated with higher rates of metastasis in breast cancer (11,14). Furthermore, PLK4 knockdown significantly inhibits invasion of neuroblastoma cells (13). However, the exact function of PLK4 in CRC development and metastasis remains unclear and requires further investigation.

Epithelial-mesenchymal transition (EMT) is a developmental process, which enhances invasion and metastasis in several types of cancer (16). During EMT, E-cadherin, and occludin are the most commonly detected epithelial markers, and N-cadherin, vimentin and snail are the most commonly detected mesenchymal markers, respectively. The multi-step process of EMT involves numerous regulatory mechanisms, including activation of the Wnt/β-catenin signaling pathway (17,18). However, to the best of our knowledge, the association between PLK4 and EMT in CRC has yet to be investigated.

To determine the expression pattern of PLK4 in CRC, the expression status of PLK4 was profiled in CRC tissues. Furthermore, its role in colorectal carcinogenesis was explored by silencing the PLK4 expression in CRC cell lines. Finally, it was revealed that PLK4 promoted proliferation and invasion of CRC cells through the Wnt/β-catenin signaling pathway.

Materials and methods

Patients and tissue specimens. CRC tissues were obtained from patients who received surgical resection at the Tongji Hospital of Huazhong University of Science and Technology (HUST) (Wuhan, China) between January 2014 and December 2016. All patients were diagnosed by two pathologists. Briefly, 39 paired freshly frozen CRC and corresponding noncancerous tissues were immersed in RNAlater overnight at 4˚C for reverse transcription-quantitative polymerase chain reaction (RT-qPCR), or were lysed using radioimmunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, Shanghai, China) at 4˚C for western blotting. All patients provided written informed consent prior to study enrollment. This study was approved by the Ethics Committee of Tongji Hospital, HUST and the study was conducted according to the principles of the Declaration of Helsinki.

Online PLK4 expression analysis. The transcript levels of PLK4 in CRC samples were analyzed using the online UALCAN program: http://ualcan.path.uab.edu (19). Sample data from The Cancer Genome Atlas (TCGA) database were analyzed using the program. A total of 41 normal tissues and 286 primary tumour tissues were included in the analysis.

Cell culture. The SW48, SW480, HCT116, SW620, RKO, LoVo and Caco-2 human CRC cell lines were purchased from American Type Culture Collection (Manassas, VA, USA) and were maintained in our laboratory. The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in an atmosphere containing 5% CO_2.

Plasmid construction and lentivirus infection. The pLKO.1-TRC cloning vector (cat. no. 10878; from Addgene, Inc., Cambridge, MA, USA) was a gift from Professor David Root (Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, MA, USA). pMD2.G and psPAX2 (cat. nos. 12259 and 12260; Addgene, Inc.) were gifts from Professor Didier Trono (School of Life Sciences, EPFL, Lausanne, Switzerland). cDNA encoding full-length human PLK4 was obtained from Addgene, Inc. (cat. no. 41165) and subcloned into the *Bam*HI/*Eco*RI sites of the pCDNA3.1 vector (cat. no. V79020; Invitrogen; Thermo Fisher Scientific, Inc.), for overexpression by transient transfection (2 μg, 36 h) into the HCT116 and SW480 cells ($1x10^6$ cells/well in 6-well plates) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Short hairpin (sh)RNAs against human PLK4 were subcloned into the *Age*I/*Eco*RI sites of the pLKO.1 vector. The sequences were as follows: shRNA#1, TRCN0000002371; sense 5'-CCGGCGTTGGTTGCTCACAGGTTAACTCGAGTTAACCTGTGAGCAACCAACGTTTTT-3'; shRNA#2, TRCN0000121260: sense 5'-CCGGGACCTTATTCACCAGTTACTTCTCGAGAAGTAACTGGTGAATAAGGTCTTTTTG-3'; and control vector (cat. no. SHC005). shRNA and control vectors were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). For plasmid transfection, 293T cells (China Center for Type Culture Collection, Wuhan, China) were plated into 6-cm dishes ($2x10^6$ cells) and were cotransfected with pLKO.1-shRNA plasmids (1 μg) and virus packaging plasmids (pMD2.G, 0.25 μg; psPAX2, 0.75 μg) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) or Xtreme HP (Roche Diagnostics, Basel, Switzerland) at 37˚C. A total of 8 h post-transfection, cells were transferred to fresh medium containing 10% FBS and were incubated for 48-72 h at 37˚C. Collected lentiviral supernatants were filtered through a 0.45-μm filter (Pall Life Sciences, Port Washington, NY, USA), concentrated with Centricon Plus 70 (Merck KGaA), according to the manufacturer's protocol, and used to infect LoVo and SW620 cells ($1x10^6$ cells/well in 6-well plates). Lentiviruses were infected into CRC cells (multiplicity of infection=10) in the presence of polybrene (8 μg/ml). A total of 48 h post-infection, cells were selected with growth medium containing 5 μg/ml puromycin for 7 days. Knockdown/overexpression efficiency was verified by RT-qPCR or western blotting.

RT-qPCR. Total RNA was extracted from CRC cell lines using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Equal amounts of RNA were used to generate first strand cDNA using the PrimeScript® RT reagent kit (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol. qPCR amplification was conducted using the CFX96 Touch™ Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the SYBR-Green PCR Master Mix-PLUS (Toyobo Life Science, Osaka, Japan), according to the manufacturers' protocols. The qPCR experiment was performed in a volume (10 μl) comprising 1 μl cDNA, 0.6 μl primers, 3.4 μl ddH_2O and 5 μl SYBR. The cycling parameters were as follows: Initial denaturation at 95˚C for 3 min, followed by 35 cycles at 95˚C for 10 sec, 55-60˚C for 20 sec and 72˚C for 15 sec, and a final extension step at 72˚C for 5 min. The primer sequences were as follows: PLK4, forward 5'-AGACCACCCTTCGACACTGA-3', reverse 5'-GTCCTTGGC-CTCTATTGACAAA-3'; c-Myc, forward 5'-GGCTCCTGGCAAAAGGTCA-3', reverse 5'-CTGCGTAGTTGTGCTGATGT-3'; cyclin D1, forward 5'-GCTGCGAAG-TGGAAACCATC-3', reverse 5'-CCTCCTTCTGCACACATTTGAA-3'; p21, forward 5'-TGTCCGTCAGAACCCATGC-3', reverse 5'-AAAGTCGAAGTTCCATCGCTC-3'; and GADPH, forward 5'-GACAAGCTTCCCGTTCTCAG-3' and reverse 5'-GAGTCAACGGATTTGGTCGT-3'. Subsequently, the relative expression levels of each gene were analyzed using the $2^{-\Delta\Delta Cq}$ method (20). The experiments were conducted in triplicate. All primers were synthesized and purchased from Tsingke Biological Technology (Beijing, China).

Cell proliferation assay. To determine the effects of PLK4 on the viability of CRC cells, 1,000 cells/well were cultured in 96-well plates; each well contained 200 μl medium. Cell viability was

Table I. Antibodies used in the present study.

Antibody/kit name	Cat. no. and manufacturer	Application
PLK4	12952-1-AP; Proteintech Group, Inc., Chicago, IL, USA	1:100 for IHC; 1:1,000 for WB
β-catenin	#8480; Cell Signaling Technology, Inc., Danvers, MA, USA	1:100 for IHC; 1:1,000 for WB
Ki-67	ab15580; Abcam, Cambridge, UK	1:100 for IHC
pGSK3β	#5558; Cell Signaling Technology, Inc.	1:1,000 for WB
GSK3β	#12456; Cell Signaling Technology, Inc.	1:1,000 for WB
GAPDH	KC-5G4; KangChen BioTech Co., Ltd., Shanghai, China	1:10,000 for WB
p21	#2947; Cell Signaling Technology, Inc.	1:1,000 for WB
Cyclin D1	#2978; Cell Signaling Technology, Inc.	1:1,000 for WB
c-Myc	#13987; Cell Signaling Technology, Inc.	1:2,000 for WB
MMP1	ab137332; Abcam, Cambridge, MA, USA	1:1,000 for WB
MMP2	ab37150; Abcam	1:1,000 for WB
E-cadherin	ab76055; Abcam	1:1,000 for WB
N-cadherin	ab98952; Abcam	1:1,000 for WB
Occludin	13409-1-AP; Proteintech Group, Inc.	1:1,000 for WB
Snail	ab82846; Abcam	1:1,000 for WB
HRP-conjugated anti-rabbit IgG	111-035-003; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA	1:5,000 for WB
HRP-conjugated anti-mouse IgG	115-035-003; Jackson ImmunoResearch Laboratories, Inc.	1:5,000 for WB
Secondary antibody in the Envision kit (HRP, rabbit/mouse, DAB+)	Dako; Agilent Technologies, Inc., Santa Clara, CA, USA	Ready-to-use for IHC

HRP, horseradish peroxidase; IgG, immunoglobulin G; IHC, immunohistochemistry; GSK3β, glycogen synthase kinase 3β; MMP, matrix metalloproteinase; p, phosphorylated; PLK4, polo-like kinase 4; WB, western blotting.

detected using a Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's protocol. After 24, 48, 72 and 96 h, 10 μl CCK-8 assay reagent was added to each well mixed with 90 μl serum-free medium. The absorbance was measured 2 h later using a microplate reader at a test wavelength of 450 nm.

Colony formation assay. CRC cells (500/well) were cultured in 6-well plates to investigate the effects of PLK4 on the efficiency of colony formation. After 10 days, each well was washed with PBS three times at room temperature. The cells were then fixed in each well using anhydrous ethanol (99.5%) for 10 min and stained for 20 min using crystal violet dye (0.1% in PBS) at room temperature. After washing with PBS, colonies (≥50 cells/colony) in each well were manually counted using a ChemiDoc™ Imaging system (Bio-Rad Laboratories, Inc.). The experiments were repeated three times.

Wound scratch assay. A confluent monolayer of CRC cells (confluence, 95%) was cultured overnight and a scratch was introduced using a pipette tip. Cell migration was recorded under a phase contrast microscope (Nikon Digital Eclipse C1 system; magnification, x40; Nikon Corporation, Tokyo, Japan) with white light at 0 and 24 h after scratch generation. Images of five random fields across three replicate wells were captured for semi-quantification using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Transwell cell migration and invasion assays. Cell migration assays were performed using a 24-well Transwell plate (pore size, 8 μm; Corning Incorporation, Corning, NY, USA), according to the manufacturer's protocol. For the Matrigel invasion assay, filters were precoated with 50 μl 1:4 mixture of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and DMEM for 4 h at room temperature. Briefly, for invasion and migration assays, culture medium containing 10% FBS was added to the lower chambers and aliquots of $5x10_4$ cells in 100 μl serum-free medium were seeded into the upper chambers. After a 24 h incubation at 37˚C, non-migrated or non-invaded cells were removed by scraping the upper surface of the membranes with a cotton swab. Cells on the lower surface of the membranes were fixed with 4% paraformaldehyde at room temperature for 15 min and stained with 0.1% crystal violet at room temperature for 20 min. Cell numbers were counted under an optical microscope. Each experiment was repeated at least three times.

Immunohistochemistry. Surgically excised tumor specimens and xenograft tumour were fixed in 4% neutral formalin at room temperature for 24 h, embedded in paraffin and cut into 4 μm sections. The sections were then deparaffinized in xylene, rehydrated in a graded alcohol series and treated with boiling 0.01 mol/l citrate buffer 15 min for antigen retrieval. Endogenous peroxidase activity was blocked with hydrogen peroxide (0.3%) at room temperature for 15 min,

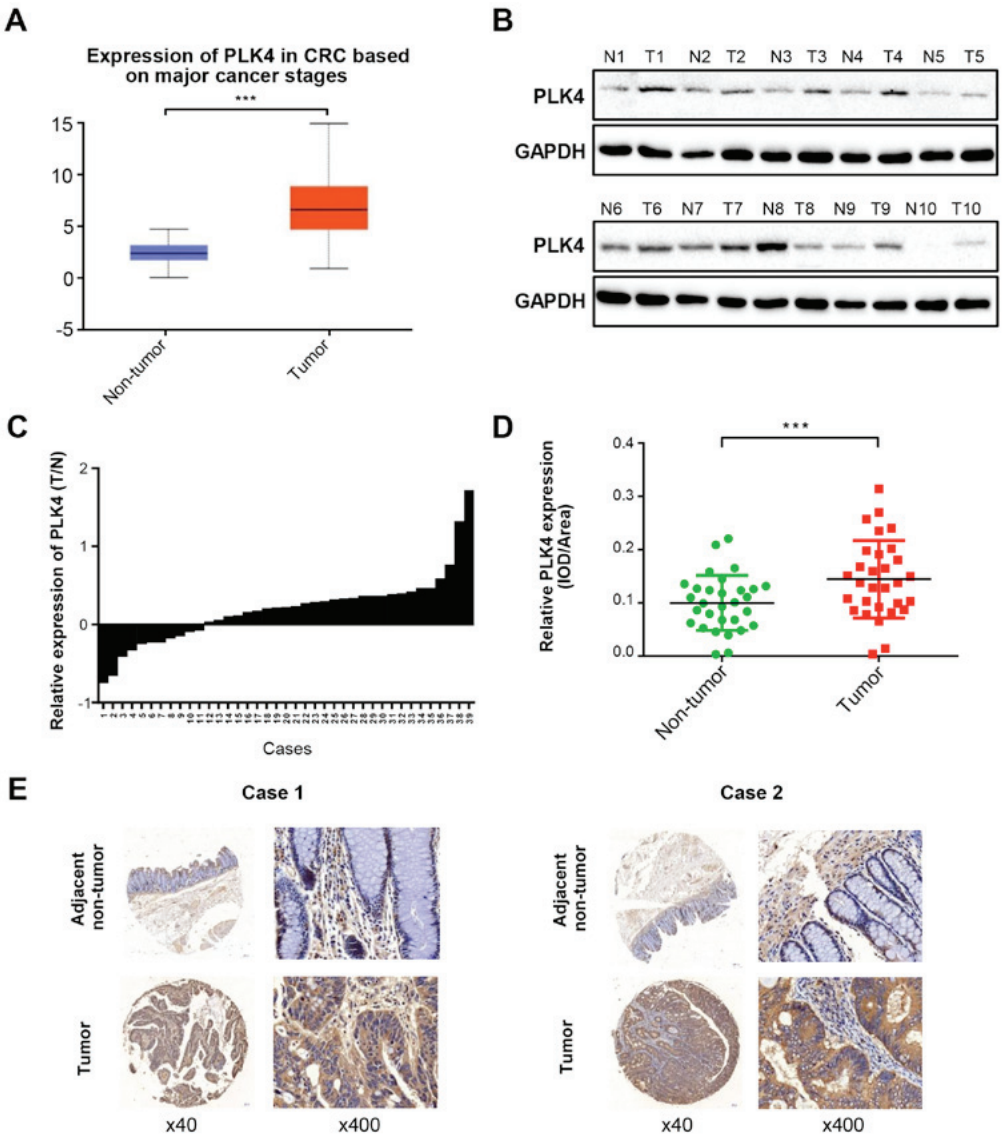

Figure 1. PLK4 expression was significantly increased in CRC tissues. (A) Expression levels of PLK4 were analyzed in CRC and normal colon tissues from The Cancer Genome Atlas CRC dataset. (B) PLK4 protein expression in 39 pairs of CRC tissues via western blotting. (C) Statistical analysis of relative PLK4 expression levels in CRC tissues and normal tissues; 64.1% of patients exhibited increased expression of PLK4 compared with normal tissues. (D) Statistical analysis of relative PLK4 expression levels in CRC tissues and normal tissues; these findings indicated that PLK4 was significantly upregulated in tumor tissues. (E) Immunohistochemical analysis of PLK4 expression in 39 paired CRC tissues. Representative images of PLK4 staining in CRC and normal colon tissues are presented. ***P<0.001. CRC, colorectal cancer; IOD, integrated optical density; PLK4, polo-like kinase 4.

and the sections were incubated with 5% bovine serum albumin (R&D Systems, Minneapolis, MN, USA) at 37˚C for 45 min to reduce non-specific binding. Immunostaining with PLK4 rabbit polyclonal antibodies (Table I) was carried out at 4˚C for 16 h, followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody from the Envision kit (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA; Table I) for 45 min at room temperature. Antibody binding was detected by DAB (Dako; Agilent Technologies, Inc.), according to manufacturer's protocol, at room temperature for 1 min and the reaction was terminated by immersion of tissue sections in distilled water once brown staining appeared. Tissue sections were counterstained with 1% hematoxylin at room temperature for 3 min and dehydrated in a graded series of ethanol. The xenograft tumour tissues were also immunostained with PLK4, Ki-67 and β-catenin primary antibodies (Table I) using the same protocol. Immunohistochemical scores were obtained by multiplying the percentage score to the intensity score of positively stained cells; images of representative fields were obtained from Nikon Digital ECLIPSE C1 microscope (Nikon Corporation) (21). Analysis was independently performed by two certified pathologists, which were blinded to the clinical and demographic characteristics of the patients. The expression status represents the average of the independent readings. An overall score of >6 and ≤6 was defined as high and low expression, respectively.

Western blotting. Cells were lysed with RIPA buffer (Beyotime Institute of Biotechnology) containing a protease inhibitor mixture on ice for 30 min. Cell lysates were then quantified using the bicinchoninic acid protein assay prior to separation (20 μg/lane) by 10% SDS-PAGE and transfer to polyvinylidene fluoride membranes (Roche Diagnostics). The

Table II. Association between PLK4 levels in CRC tissues and clinicopathological features (n=39).

Clinical characteristic	n (%)	PLK4 expression		P-value
		Low (%)	High (%)	
Overall	39	14 (35.9)	25 (64.1)	
Age				
<65 years	27 (69.2)	11 (28.2)	16 (41.0)	0.283
≥65 years	12 (30.8)	3 (7.7)	9 (23.1)	
Sex				
Male	18 (46.2)	5 (12.8)	13 (33.3)	0.261
Female	21 (53.9)	9 (23.1)	12 (30.8)	
Tumor size				
≤5 cm	16 (41.0)	9 (23.1)	7 (17.9)	0.031
>5 cm	23 (59.0)	5 (12.8)	18 (46.2)	
Lymph node metastasis				
Absent	15 (38.5)	9 (23.1)	6 (15.4)	0.016
Present	24 (61.5)	5 (12.8)	19 (48.7)	
Location				
Rectum	19 (48.7)	8 (20.5)	11 (28.2)	0.325
Colon	20 (51.3)	6 (15.4)	14 (35.9)	
TNM stage (AJCC)				
Stage I-II	16 (41.0)	11 (28.2)	5 (12.8)	0.001
Stage III	23 (59.0)	3 (7.7)	20 (51.3)	

AJCC, American Joint Committee on Cancer; PLK4, polo-like kinase 4.

membranes were blocked with 5% non-fat milk at 37˚C for 1 h and were incubated with primary antibodies (Table I) at 4˚C overnight. Subsequently, the membranes were incubated with HRP-conjugated goat anti-rabbit or goat anti-mouse immunoglobulin G secondary antibodies (Table I) for at 37˚C for 1 h. Finally, the enhanced chemiluminescence detection system (Bio-Rad Laboratories, Inc.) was used for visualization. Image Lab™ 3.0 software (Bio-Rad Laboratories, Inc.) was used to semi-quantify blots.

Animal study. A total of 15 male BALB/c nude mice (age, 4 weeks; weight, 18-19 g) were purchased from the Animal Center of East China Normal University (Shanghai, China), after signing the animal-raising agreement. The mice were maintained under the following conditions: Room temperature, 20-26˚C; humidity, 40-60%; 12-h light/dark cycle; free access to food and water. Mice were randomly divided into three groups (n=5/group). After anesthesia, mice in the control group were injected with $2x10^6$ LoVo cells transduced with the empty vector in 100 μl DMEM, whereas mice in the treatment groups were injected with $2x10^6$ LoVo cells transduced with PLK4 knockdown viruses in 100 μl DMEM. Tumor length (L) and width (W) were manually monitored using a Vernier caliper twice a week, and tumor volume (V) was calculated according to the following equation: V (mm^3) = 0.5 x L (mm) x W^2 (mm^2). A total of 4 weeks post-injection and after the last tumor volume was measured all mice were sacrificed. For sacrifice, the mice were placed into a sealed container and the concentration of CO_2 in the container was increased gradually (flow rate, 20% of the chamber volume/min). Subsequently, tumor tissues were removed and tumor weight was measured; in the present study, each mouse bore a single tumor, the largest weight of which was 0.35 g, and the percentage of all tumors to the body weight of the animals was <1.5%. All of the animal experiments met the National Institutes of Health (NIH) guidelines (NIH publication no. 86-23, revised 1985) (22) and the animal studies were approved by the Committee on the Ethics of Animal Experiments of the Tongji Medical College, HUST.

Statistical analysis. All experiments were repeated at least twice with consistent results. If the variance was homogenous, the difference between two groups was analyzed using Student's t-test, and the difference between more than two groups was analyzed by one-way analysis of variance. If the variance was not homogeneous, the difference between two groups was analyzed using the Mann-Whitney U test, and the difference between more than two groups was analyzed by Kruskal-Wallis H test. The least-significant difference post hoc test was used to compare datasets containing multiple groups. Categorical data in Table II were analyzed by χ^2 test or Fisher's exact test. All statistical analyses were performed using SPSS Statistics version 20 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

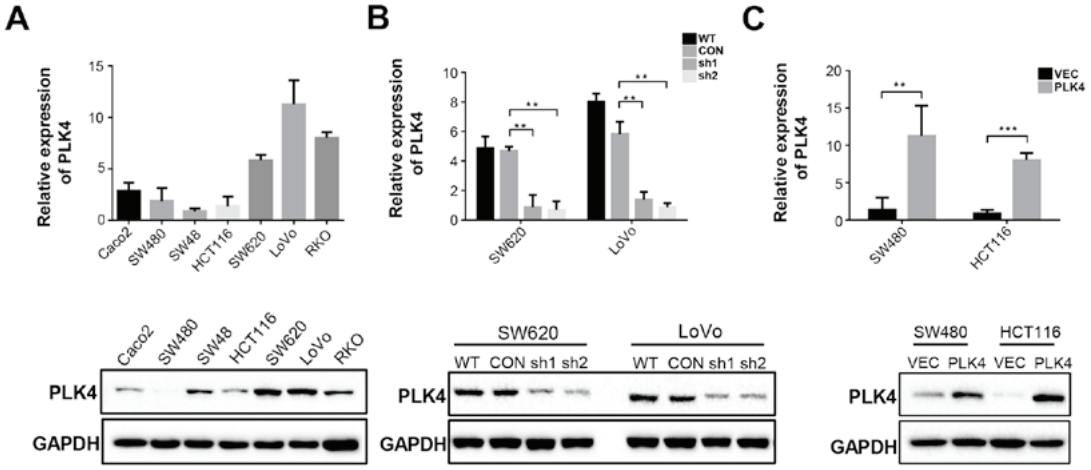

Figure 2. Manipulation of PLK4 expression in CRC cell lines. (A) Protein (bottom panel) and mRNA (top panel) expression levels of PLK4 in seven CRC cell lines. GAPDH was used as an internal control. (B) SW620 and LoVo cells were infected with PLK4 sh1 and sh2 lentiviruses or CON shRNA lentivirus. PLK4 mRNA (top panel) and protein (bottom panel) expression levels were analyzed 48 h post-viral infection. (C) Overexpression of PLK4 in HCT116 and SW480 cells was induced by plasmid transfection (4 μg per 6-cm dish). PLK4 mRNA (top panel) and protein (bottom panel) expression levels were analyzed after 48 h. **P<0.01, ***P<0.001. CON, control; CRC, colorectal cancer; PLK4, polo-like kinase 4; sh/shRNA, short hairpin RNA; VEC, empty vector; WT, wild type (uninfected cells).

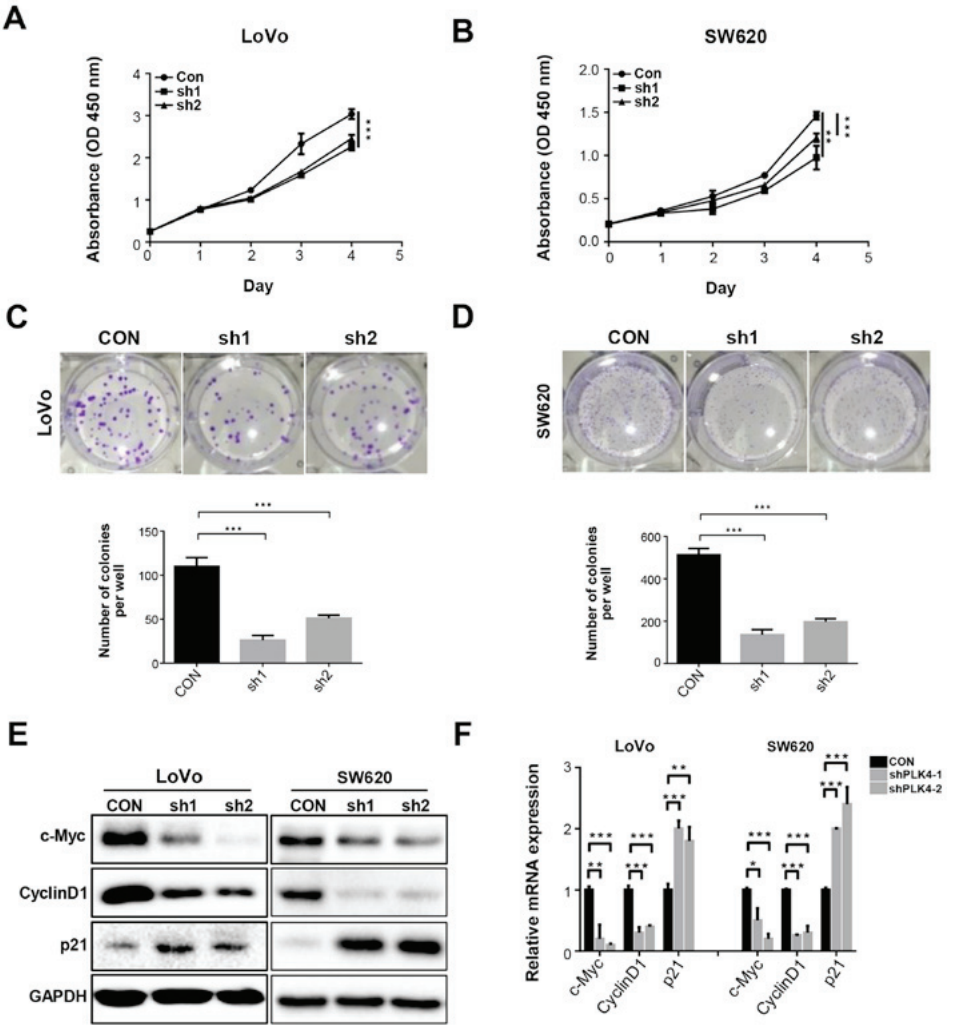

Figure 3. Knockdown of PLK4 inhibits proliferation and colony formation in colorectal cancer cells. (A and B) Effects of PLK4 knockdown on SW620 and LoVo cell proliferation, as evaluated by Cell Counting Kit-8 assays. (C and D) Effects of knockdown on SW620 and LoVo PLK4 cell colony formation. (E) Western blot analysis of cyclin D1, c-Myc and p21 in SW620 and LoVo cells with PLK4 knockdown. (F) Reverse transcription-quantitative polymerase chain reaction analysis of cyclin D1, c-Myc and p21 mRNA expression in SW620 and LoVo cells with PLK4 knockdown. Results are presented as the means ± standard error of the mean of triplicate repeats from three independent experiments. *P<0.05, **P<0.01, ***P<0.001. CON, control; OD, optical density; PLK4, polo-like kinase 4; sh/shRNA, short hairpin RNA.

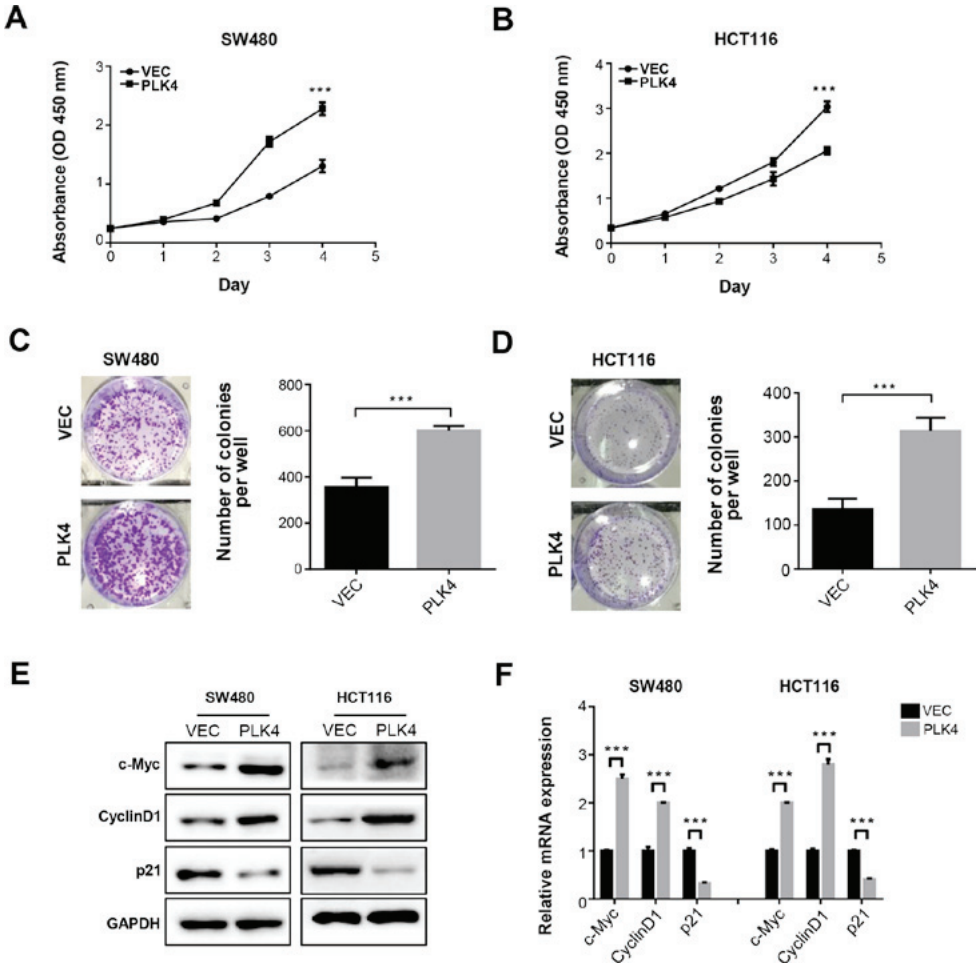

Figure 4. Overexpression of PLK4 promotes proliferation and colony formation in colorectal cancer cells. (A and B) Effects of PLK4 overexpression on SW480 and HCT116 cell proliferation, as evaluated by Cell Counting Kit-8 assays. (C and D) Effects of PLK4 overexpression on SW480 and HCT116 cell colony formation assay. (E) Western blot analysis of cyclin D1, c-Myc and p21 in SW480 and HCT116 cells with PLK4 overexpression. (F) Reverse transcription-quantitative polymerase chain reaction analysis of cyclin D1, c-Myc and p21 mRNA expression in SW480 and HCT116 cells with PLK4 overexpression. Results are presented as the means ± standard error of the mean of triplicate repeats from three independent experiments. ***P<0.001. OD, optical density; PLK4, polo-like kinase 4; VEC, empty vector.

Results

PLK4 expression is significantly upregulated in CRC tissues and is associated with clinicopathological features. To evaluate the expression status of PLK4 in human CRC tissues, UALCAN (http://ualcan.path.uab.edu/) was used to analyze the microarray dataset from TCGA; the results revealed that the mRNA expression levels of PLK4 were upregulated in the majority of tumor tissues compared with in adjacent normal tissues (Fig. 1A). Subsequently, 39 paired CRC and adjacent normal tissues were analyzed by western blotting. The results demonstrated that the protein expression levels of PLK4 were higher in the majority of CRC tissues compared with in paired noncancerous colorectal tissues (Fig. 1B and C). Furthermore, immunohistochemistry was used to assess the protein expression levels of PLK4 in 39 paraffin-embedded CRC specimens; 64.1% (25/39) of CRC samples exhibited high PLK4 expression, whereas the remaining 35.9% (14/39) of samples exhibited low PLK4 expression (Fig. 1D and E). The association between PLK4 protein expression and clinicopathological parameters was then analyzed. Upregulation of PLK4 was significantly associated with tumor size (≤5 cm vs. >5 cm; P=0.031), lymph node metastasis (absent vs. present; P=0.016) and TNM stage (I-II vs. III-IV; P=0.001). However, no significant association was observed between PLK4 expression and the other clinicopathological factors, including sex and age (Table II).

Expression of PLK4 is upregulated in CRC cells, and can be regulated by shRNA and plasmids. To detect the expression levels of PLK4 in CRC cells, seven CRC cell lines (Caco2, SW480, SW48, HCT116, SW620, LoVo and RKO) were analyzed by RT-qPCR and western blot analysis; SW620 and LoVo exhibited high endogenous PLK4 expression, whereas HCT116 and SW480 exhibited low endogenous PLK4 expression (Fig. 2A). Subsequently, PLK4 was knocked down in SW620 and LoVo cells using shRNA-PLK4 and was overexpressed in HCT116 and SW480 cells using plasmids. Knockdown/overexpression efficiency was confirmed by RT-qPCR and western blot analysis (Fig. 2B and C).

PLK4 significantly increases CRC cell proliferation and colony formation in vitro. CCK-8 and colony formation assays were conducted to determine the effects of PLK4 on CRC cell

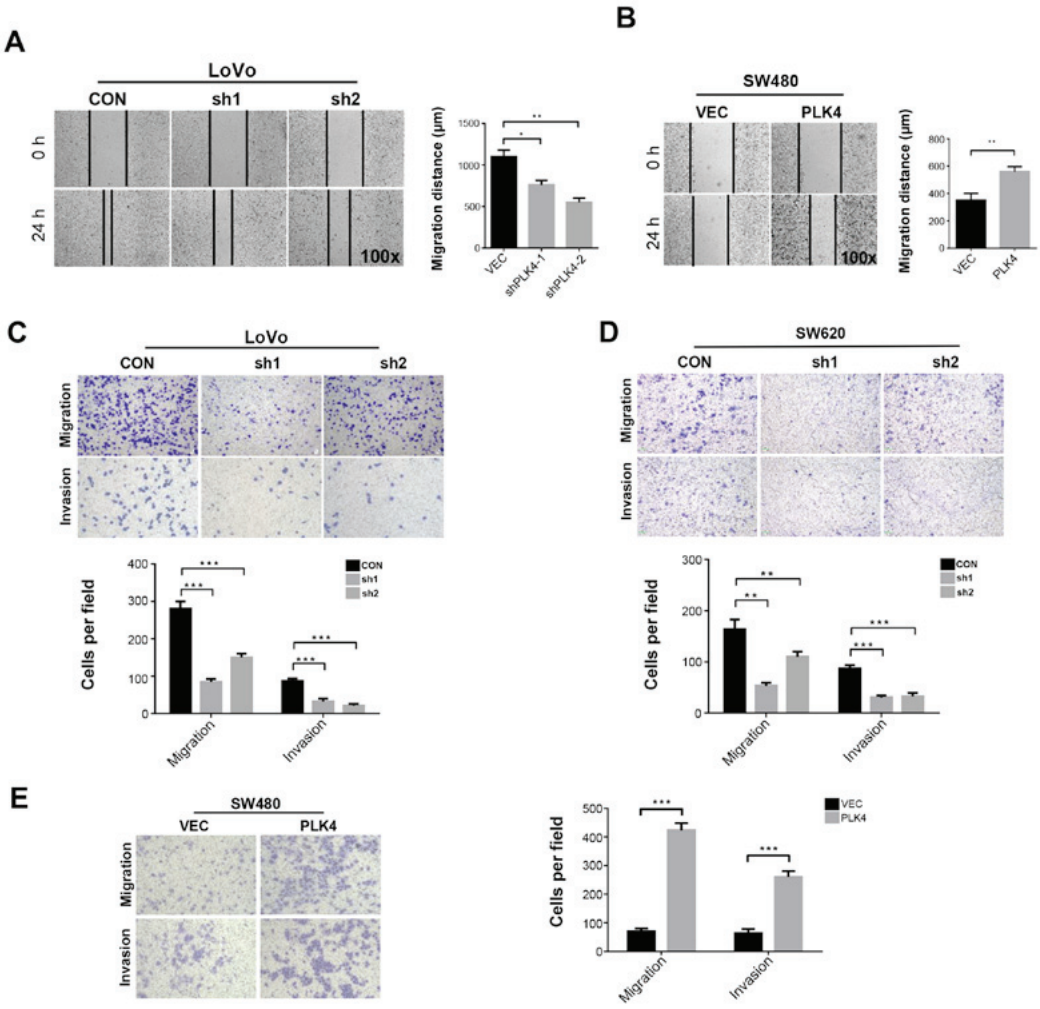

Figure 5. PLK4 promotes migration and invasion of colorectal cancer cells. (A) Effects of PLK4 knockdown on LoVo cell migration, as evaluated by wound scratch assay. (B) Effects of PLK4 overexpression on SW480 cell migration, as evaluated by wound scratch assay. (C) Effects of PLK4 knockdown on LoVo cell migration and invasion, as evaluated by Transwell assay. (D) Effects of PLK4 knockdown on SW620 cell migration and invasion, as evaluated by Transwell assay. (E) Effects of PLK4 overexpression on SW480 cell migration and invasion, as evaluated by Transwell assay. Results are presented as the means ± standard error of the mean of triplicate repeats from three independent experiments. *P<0.05, **P<0.01, ***P<0.001. CON, control; CRC, colorectal cancer; PLK4, polo-like kinase 4; sh/shRNA, short hairpin RNA; VEC, empty vector.

proliferation (Figs. 3 and 4). It was revealed that knockdown of PLK4 significantly inhibited the proliferation of SW620 and LoVo cells (Fig. 3A and B). Conversely, proliferation was promoted in SW480 and HCT116 cells overexpressing PLK4 (Fig. 4A and B). Consistently, the results of the colony formation assay demonstrated that PLK4 shRNA-infected SW620 and LoVo cells exhibited reduced colony formation (Fig. 3C and D), whereas PLK4-overexpressing plasmid-transfected SW480 and HCT116 cells exhibited opposite effects (Fig. 4C and D). Analysis of the mRNA and proteins involved in cell proliferation and cell cycle progression indicated that cyclin D1 and c-Myc expression was downregulated, whereas p21 expression was upregulated by PLK4 knockdown (Fig. 3E and F), whereas PLK4 overexpression had the opposite effects (Fig. 4E and F).

Regulation of PLK4 affects the migration and invasion of CRC cells in vitro. Wound-healing and Transwell assays were conducted to detect migration and invasion of LoVo and SW480 cells. The results of the wound-healing assay demonstrated that the wound area was wider in LoVo cells in which PLK4 was knocked down (Fig. 5A). Conversely, the wound area was narrower in SW480 cells transfected with the PLK4-overexpressing plasmid (Fig. 5B). The results of the Transwell assay revealed that the number of cells that traversed the membrane and Matrigel was significantly reduced by PLK4 knockdown in LoVo and SW620 cells (Fig. 5C and D), whereas overexpression of PLK4 in SW480 cells increased the number of cells that traversed the membrane and Matrigel (Fig. 5E). These findings indicated that PLK4 may promote invasion and metastasis of CRC cells.

PLK4 facilitates EMT and activates the Wnt/β-catenin pathway in CRC cells. To determine whether PLK4 was associated with EMT in CRC cells, the present study examined the expression levels of EMT-associated proteins, including MMP1, MMP2, N-cadherin, occludin and snail, by western blotting. The results revealed that knockdown of PLK4 significantly increased the expression of occludin, and decreased the expression of MMP1, MMP2, N-cadherin and snail (Fig. 6). These results suggested that PLK4 may induce EMT in CRC cells. In addition, the expression levels of pGSK3β, GSK3β and β-catenin were

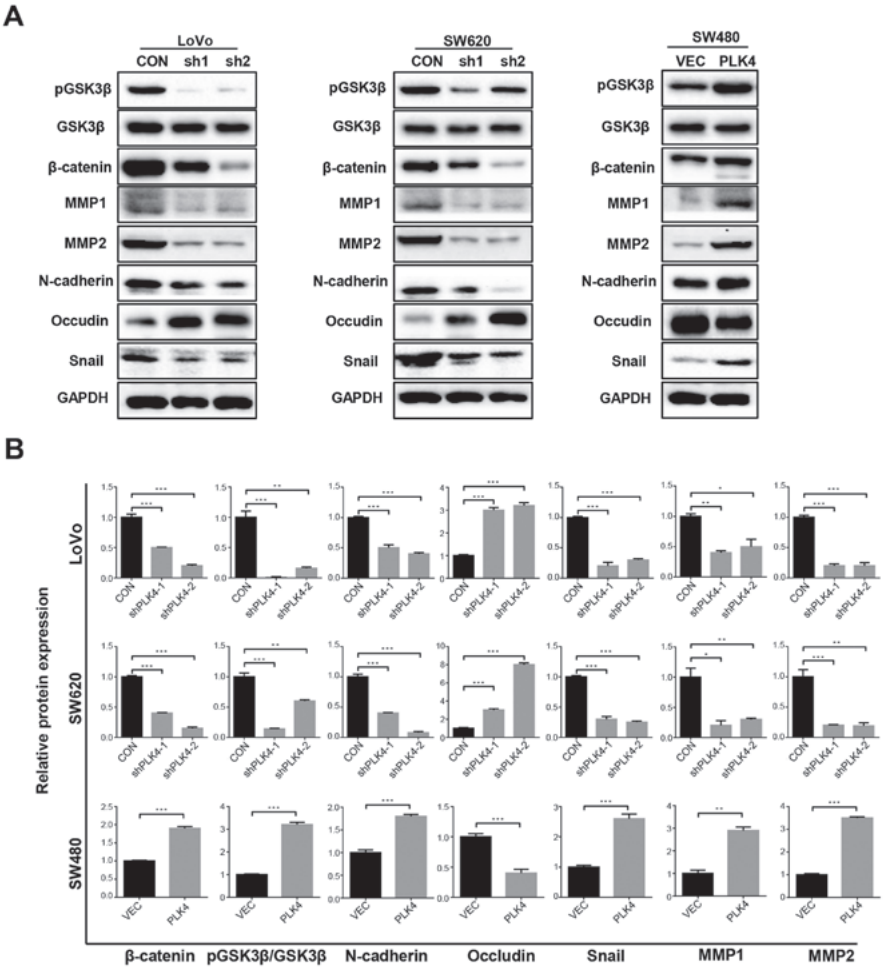

Figure 6. PLK4 promotes activation of the Wnt/β-catenin signaling pathway and epithelial-mesenchymal transition in colorectal cancer cells. (A) Expression levels of pGSK3β, β-catenin, MMP1, MMP2, N-cadherin, occludin and snail in LoVo, SW620 and SW480 cells following PLK4 knockdown and overexpression were detected using western blot analysis. (B) Relative protein expression levels of β-catenin, N-cadherin, occludin, snail, MMP1 and MMP2 were semi-quantified in LoVo, SW620 and SW480 cells. Data are presented as the means ± standard deviation from three independent experiments. **P<0.01 and ***P<0.001. CON, control; GSK3β, glycogen synthase kinase 3β; MMP, matrix metalloproteinase; p, phosphorylated; PLK4, polo-like kinase 4; sh/shRNA, short hairpin RNA; VEC, empty vector.

detected in LoVo, SW620 and SW480 cells. It was revealed that the protein expression levels of pGSK3β and β-catenin were increased when PLK4 was overexpressed, but were decreased when PLK4 was knocked down. However, no significant differences were detected in GSK3β expression among the groups. These findings suggested that PLK4 promoted activation of the Wnt/β-catenin pathway in CRC (Fig. 6).

Downregulation of PLK4 significantly suppresses CRC tumorigenesis in vivo. To verify the effects of PLK4 expression on tumorigenesis *in vivo*, mice were transplanted with LoVo cells transduced with a PLK4 knockdown virus or empty vector. Tumor growth was markedly decreased in the shPLK4 groups compared with in the vector group (Fig. 7A). A total of 4 weeks post-inoculation, tumor volume (Fig. 7B) and weight (Fig. 7C) were significantly smaller in the shPLK4 groups compared with in the vector group. Furthermore, Ki67-positive signals in the xenograft tumors were decreased in the shPLK4 groups (Fig. 7D). To further detect whether PLK4 was associated with EMT, the expression levels of EMT-associated proteins were detected in tumor tissues using western blot analysis. Following PLK4 knockdown, it was revealed that the expression levels of the epithelial markers E-cadherin and occludin were increased, whereas the expression levels of the mesenchymal markers N-cadherin and snail were decreased (Fig. 7F and G). In addition, the expression levels of pGSK3β, GSK3β and β-catenin were detected. The results demonstrated that lower levels of pGSK3β and β-catenin were detected in the shPLK4 group (Fig. 7E and G). Taken together, these results indicated that PLK4 may serve a crucial role in CRC progression and may activate the Wnt/β-catenin pathway *in vivo*.

Discussion

The present study indicated that PLK4 was highly expressed in CRC tissues. Stepwise investigation demonstrated that PLK4 activated the Wnt/β-catenin pathway to promote cell proliferation and invasion in CRC. These findings suggested that PLK4 may be a novel biomarker for patients with CRC.

Abnormal PLK4 expression may regulate centroid replication, abnormal mitosis, centrosome amplification (CA) and chromosomal instability (CIN) (6,23,24), which are common causes of cancer development (23,25,26), thus we speculated that PLK4 may be involved in CRC development. Furthermore,

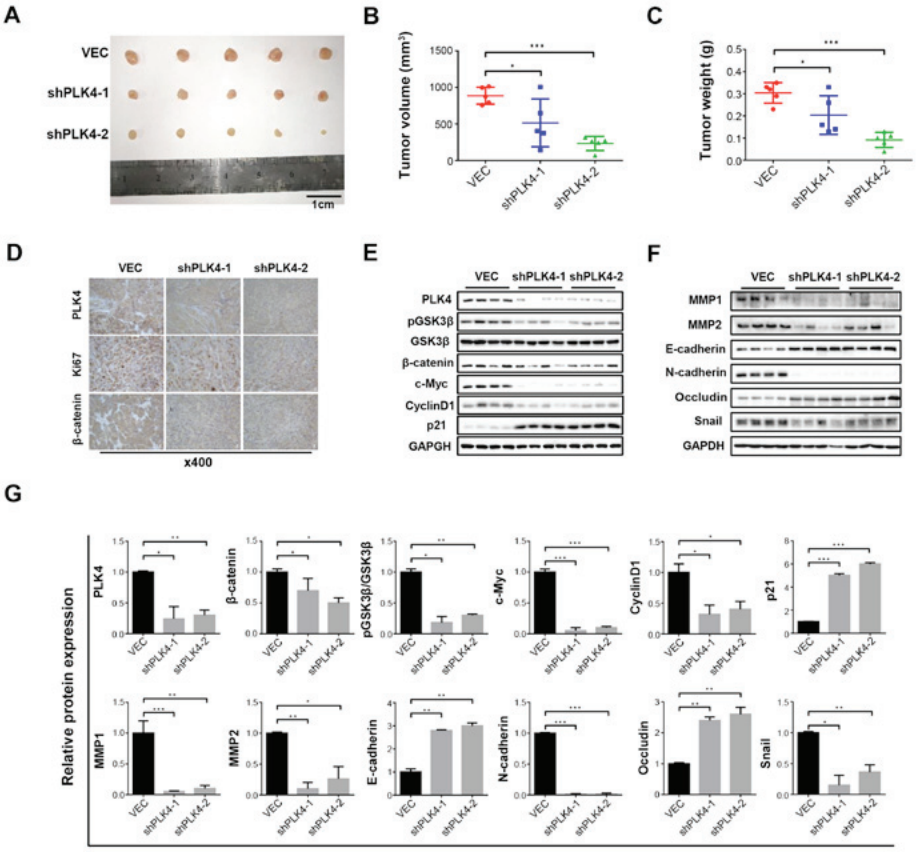

Figure 7. Knockdown of PLK4 inhibits tumor growth *in vivo*. (A) Representative images of isolated tumors in the control group (first row), shPLK4-1 group (second row) and shPLK4-2 group (third row). (B) Xenograft tumor volume was smaller in the shPLK4 groups compared with in the control group. (C) Xenograft tumor weight was reduced in the shPLK4 groups compared with in the control group. (D) Ki67-positive cells in subcutaneous xenograft tumor tissues developed from CRC cells. (E and F) Relative protein expression levels of pGSK3β, β-catenin, c-Myc, cyclin D1, p21 and epithelial-mesenchymal transition markers were examined in CRC xenograft tissues. (G) Relative protein expression levels of PLK4, β-catenin, pGSK3β, c-Myc, cyclin D1, p21, MMP1, MMP2, E-cadherin, N-cadherin, occludin and snail were semi-quantified. Results are presented as the means ± standard error of the mean of triplicate repeats from three independent experiments. *P<0.05, **P<0.01, ***P<0.001. CRC, colorectal cancer; GSK3β, glycogen synthase kinase 3β; MMP, matrix metalloproteinase; p, phosphorylated; PLK4, polo-like kinase 4; sh/shRNA, short hairpin RNA; VEC, empty vector.

it has been reported that PLK4 is overexpressed in various types of tumor (13-15) and is closely associated with the prognosis of patients with cancer. Furthermore, it has been confirmed that the mRNA expression levels of PLK4 are associated with breast cancer aggression and resistance to traditional therapy (14). Furthermore, Kazazian *et al* indicated that PLK4 enhances migration and invasion of HeLa and U2OS cells (11). PLK4 also facilitates aggressiveness of neuroblastoma, and is correlated with adverse clinical features and poor survival (13). These studies indicated that PLK4 may be an oncogenic factor in these cancer types, and may promote tumor development and progression. Conversely, knockdown of PLK4 inhibits cell apoptosis, and low PLK4 expression is associated with poor prognosis in hepatocellular carcinoma (HCC) (27). These findings suggested that PLK4 may be a tumor suppressor in HCC; these discrepancies in findings suggested that PLK4 may exert tumour promoter and suppressor functions according to cancer type.

Consistent with the findings of previous studies, PLK4 expression was upregulated in human CRC tissues in the present study. Furthermore, PLK4 promoted the proliferation and invasion of CRC cells. PLK4 also affected the expression of several EMT-associated proteins; N-cadherin and snail were upregulated, and occludin was downregulated in cells overexpressing PLK4, thus suggesting a novel mechanism underlying the role of PLK4 in cancer. The present study investigated the function of PLK4 and suggested that it may act as an oncogene in CRC. Previous studies revealed that PLK4 manipulates numerous signaling pathways, including the transforming growth factor β and epidermal growth factor receptor signaling pathways. In addition, the Wnt/β-catenin pathway is a critical mediator of carcinogenic signals in various tumor types (17,28-30). The Wnt/β-catenin signaling pathway induces the EMT process and inhibits the transcription of E-cadherin, which is commonly considered an activator of cancer progression (31-33). β-catenin directly influences the biological characteristics of cells, and has been reported to downregulate E-cadherin expression, and to promote EMT-like transition and invasiveness in carcinoma cells (34). In addition, activation of the Wnt/β-catenin pathway contributes to EMT through upregulation of EMT-associated factors, including snail and slug (34-36). In the present study, the expression levels of β-catenin were significantly increased in response to PLK4 overexpression in CRC cells. These findings indicated that PLK4 may mediate EMT in CRC cells via its effects on the Wnt/β-catenin signaling pathway, ultimately improving the migratory and invasive potential of the cells.

In conclusion, the present study demonstrated that PLK4 promoted the proliferative and invasive phenotype of CRC

cells via the Wnt/β-catenin signaling pathway. These findings suggested that PLK4 may serve as a biomarker or a target for treatment of patients with CRC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ZL, ZZ and BZ designed the experiments. ZL performed experiments and generated the data. HZ and KD collected clinicopathological data. PF, QH, YQ, JS, LC, HL and XC analyzed the results. HZ prepared the panels for Fig. 1, and KD prepared the panels for Figs. 2-7; KD also assembled the tables. ZZ and BZ wrote the manuscript. All authors reviewed the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Tongji Hospital, HUST. Written informed consent was obtained from each patient. Animal studies were approved by the Committee on the Ethics of Animal Experiments of the Tongji Medical College, HUST.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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