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Knockdown of PFTK1 Protein Kinase 1 (PFTK1) Inhibits Proliferation, Invasion, and EMT in Colon Cancer Cells

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PFTK1 is a member of the cyclin-dependent kinase (CDK) family and is upregulated in many types of tumors. However, its expression and role in colon cancer remain unclear. In this study, we aimed to investigate the expression and function of PFTK1 in colon cancer. Our results showed that PFTK1 was highly expressed in colon cancer cell lines. The *in vitro* experiments demonstrated that knockdown of PFTK1 inhibited the proliferation, migration, and invasion of colon cancer cells as well as the epithelial-to-mesenchymal transition (EMT) progress. Furthermore, knockdown of PFTK1 suppressed the expression of Shh as well as Smo, Ptc, and Gli-1 in colon cancer cells. Taken together, these results suggest that knockdown of PFTK1 inhibited the proliferation and invasion of colon cancer cells as well as the EMT progress by suppressing the Sonic hedgehog signaling pathway. Therefore, these findings reveal that PFTK1 may be a potential therapeutic target for the treatment of colon cancer.

Key words: PFTK1; Colon cancer; Invasion; Epithelial-to-mesenchymal transition (EMT)

INTRODUCTION

Colon cancer is one of the most commonly diagnosed cancers and the fourth leading cause of cancer mortality in the world. Furthermore, the incidence is increasing rapidly (1,2). Although the diagnosis and treatment of colon cancer has improved in the past years (3–5), about half of newly diagnosed colorectal cancer patients will die of this disease due to tumor recurrence and metastasis. Thus, further understanding of the molecular mechanisms of cancer progression and the development of new therapeutic tools based on these mechanisms are imperative.

Cyclin-dependent kinases (CDKs) are serine/threonine kinases that play key roles in the control of cell cycle progression and cell proliferation via their interaction with specific cyclins (6). PFTK1, also named CDK14, is a member of the cell division cycle 2 (CDC2)-related protein kinase family and contains ~140 aa N-terminal domain followed by ~300 aa kinase-conserved domain, then ~30 aa C-terminal domain (7). It is reported that PFTK1 is highly expressed in the brain, pancreas, kidneys, and ovaries. In addition, there is substantial evidence that PFTK1 is upregulated in many types of tumors (4,8,9). For example, PFTK1 was upregulated in breast cancer tissue and breast cancer lines, and knockdown of PFTK1 attenuated cell proliferation, anchorage-independent cell

growth, and cell migration and invasion in MDA-MB-231 cells (10). However, the role of PFTK1 in colon cancer has not yet been fully elucidated. In this study, we evaluated the expression and role of PFTK1 in colon cancer cells. Our results showed that the expression of PFTK1 was upregulated in colon cancer cells, and PFTK1 silencing inhibited cell proliferation, migration/invasion, and epithelial-to-mesenchymal transition (EMT) progress in colon cancer cells.

MATERIALS AND METHODS

Cell Culture

Human colon cancer cell lines (HT29, SW480, and LS174T) and normal colonic mucosa epithelial cell line (NCM460) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All of the cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO₂ atmosphere.

Real-Time Quantitative PCR Analysis

Total RNA was extracted from colon cancer cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Single-stranded cDNA was synthesized using a PrimeScript™ 1st Strand cDNA Synthesis kit (Takara,

Dalian, China) according to the supplier's instructions. Subsequently, real-time PCR was performed with SYBR Premix Ex Taq (TaKaRa Biotechnology) using an ABI Prism 7900 instrument (Life Technologies, Carlsbad, CA, USA). The following primers were used: PFTK1, 5'-CCA AGGAGTTGCTGCTTTTC-3' (sense) and 5'-GAATGAA CTCCAGGCCATGT-3' (antisense); and β -actin 5'-CCGT GAAAAGATGACCCAGATC-3' (sense) and 5'-CACAGC CTGGATGGCTACGT-3' (antisense). β -Actin was used as the internal reference gene. The relative expression levels were calculated by $2^{-\Delta\Delta Ct}$ method, and the target gene was normalized to the internal reference gene.

Western Blot

Cells were homogenized and lysed with RIPA lysis buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1 mM EDTA, 10 mM β -glycerophosphate, 2 mM sodium vanadate, and protease inhibitor), and protein concentrations were measured by using the Bradford method. Equal amounts of protein (30 μ g of protein each lane) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Boston, MA, USA). The membrane was blocked for 1 h at room temperature with SuperBlock T20 TBS Blocking Buffer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The membrane was immunoblotted with primary antibodies (anti-PFTK1, anti-E-cadherin, anti-vimentin, anti-Shh, anti-Smo, anti-Ptc, anti-Gli-1, and anti-GAPDH) (Santa Cruz Biotechnology) overnight at 4°C. The membranes were then washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies at 37°C in the blocking buffer for 1 h. The blots were developed using an enhanced chemiluminescence Western blotting detection system (Amersham Bioscience, UK).

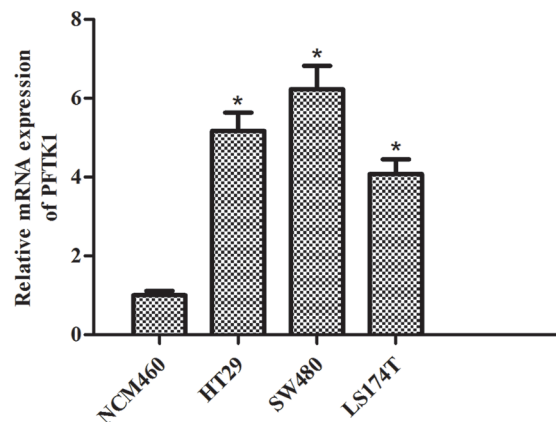
Small Interfering RNA and Cell Transfection

Scrambled siRNA and small interfering RNA (siRNA) targeting PFTK1 were purchased from Santa Cruz Biotechnology. At 60% confluency, cells were treated with plasmid or siRNA-PFTK1 using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions. The knockdown efficiency was confirmed by Western blot analysis.

Cell Proliferation Assay

Cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, the transiently transfected cells were seeded in a 96-well plate at a cell density of 1.0×10^4 cells/well and then cultured for 24, 48, and 72 h, respectively. The MTT solution (0.2 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated for an additional 4 h. Following that, 250 μ l of DMSO was added to extract the MTT formazan, and the absorbance of each

A



B

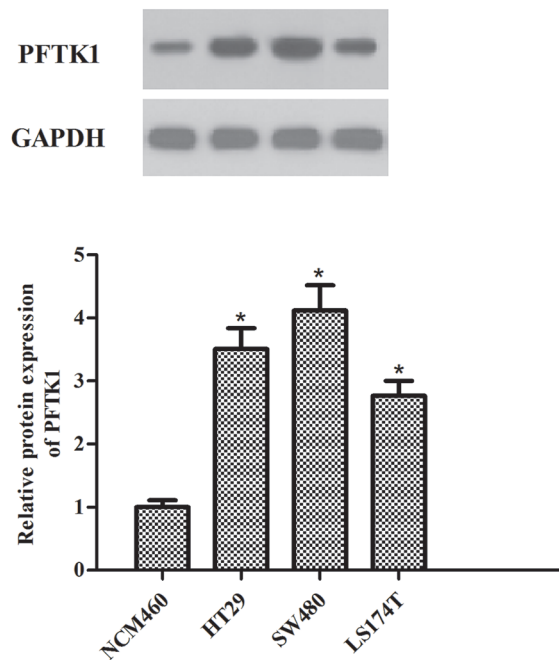


Figure 1. PFTK1 is highly expressed in colon cancer cell lines. (A) mRNA expression level of PFTK1 in colon cancer cell lines was analyzed by qRT-PCR. (B) Protein expression level of PFTK1 was detected by Western blot analysis and quantified using Image-Pro Plus 6.0 software. All the experiments were repeated at least three times. * $p < 0.05$ versus NCM460 group.

well at 490 nm was read using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

Cell Migration and Invasion Assays

For the migration assay, colon cancer cells (1.0×10^5 cells/well) transfected with siRNA-PFTK1 and mock were suspended in 200 μ l serum-free DMEM and plated on the upper chamber of the Transwell. Five hundred microliters

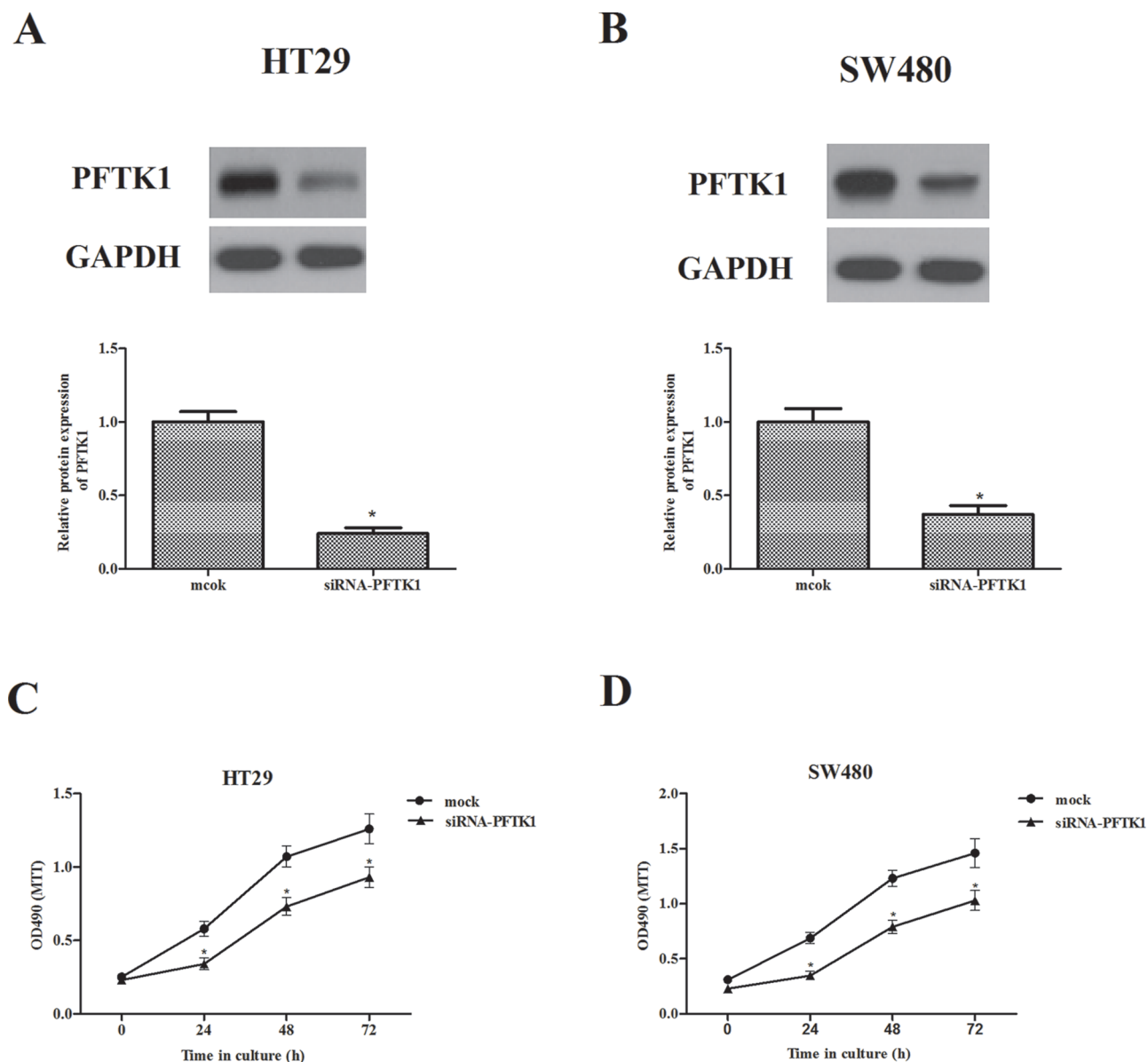


Figure 2. Knockdown of PFTK1 inhibits colon cancer cell proliferation. (A, B) HT29 and SW480 cells were infected with siRNA-PFTK1 or mock for 24 h. Western blot analysis to detect the protein expression levels of PFTK1 in different treated groups, and relative protein levels were quantified using Image-Pro Plus 6.0 software. The fold changes were presented after normalization with the control group. Data were expressed as mean \pm SD. (C, D) The effect of siRNA-PFTK1 on colon cancer cell proliferation, including HT29 and SW480 cells, was measured by MTT. All the experiments were repeated at least three times. * $p < 0.05$ versus mock.

of DMEM medium containing 10% FBS was added to the lower chamber. After incubating for 24 h at 37°C with 5% CO₂, cells were fixed in methanol for 15 min and stained with 0.05% crystal violet in PBS for 15 min. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed in PBS. Cells in the lower surface of the filter were viewed and counted under a microscope (Olympus, Tokyo, Japan).

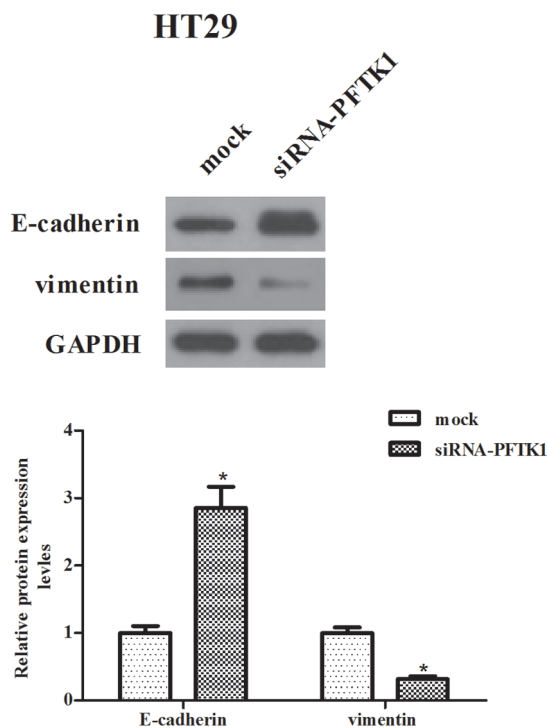
For the invasion assay, the same procedures described above were used, except that the filters were precoated

with 100 ml Matrigel (BD Biosciences, San Jose, CA, USA) at a 1:4 dilution in DMEM to form a genuine reconstituted basement membrane.

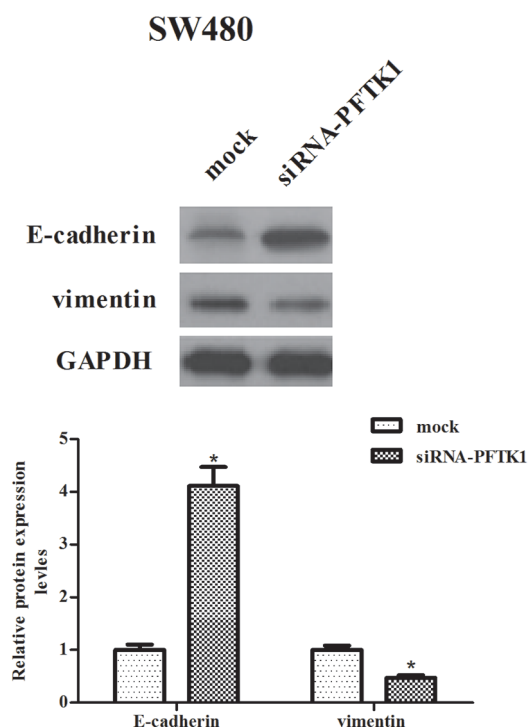
Statistical Analysis

All experiments were performed at least three times. The data were expressed as mean \pm SEM. Statistical significance of differences between groups was determined using the Student's *t*-test or the ANOVA by SPSS 22.0 software (SPSS, Inc., Chicago, IL, USA). A value of

A



B



$p < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

PFTK1 Is Highly Expressed in Colon Cancer Cell Lines

To understand whether PFTK1 is involved in the development and progression of colon cancer, endogenous PFTK1 expression was examined in human colon cancer cell lines. As shown in Figure 1A, the expression of PFTK1 mRNA in colon cancer cell lines was obviously increased, compared with the normal colonic mucosa epithelial cells. Moreover, we found that the expression of PFTK1 protein was also significantly increased in colon cancer cell lines (Fig. 1B).

Knockdown of PFTK1 Inhibits Colon Cancer Cell Proliferation

To investigate the effect of PFTK1 on cell proliferation in colon cancer cells, we downregulated PFTK1 in colon cancer cells by transfection with siRNA-PFTK1, and the expression of PFTK1 in different groups was detected by Western blot analysis. The results showed that siRNA-PFTK1 significantly decreased the expression level of PFTK1 in HT29 (Fig. 2A) and SW480 cells (Fig. 2B) when compared with the mock group. Cell proliferation was then evaluated using the MTT assay. We found that knockdown of PFTK1 greatly inhibited the proliferation in HT29 (Fig. 2C) and SW480 cells (Fig. 2D), respectively, when compared with the mock group.

Knockdown of PFTK1 Suppresses EMT in Colon Cancer Cells

In order to investigate the effect of PFTK1 on the EMT progression of colon cancer cells, we evaluated the expression of vimentin and E-cadherin by Western blot. As indicated in Figure 3A, the protein expression of vimentin was markedly inhibited, while the protein expression of E-cadherin was increased in HT29 cells transfected with siRNA-PFTK1 compared with the mock group. Similarly, knockdown of PFTK1 increased E-cadherin and decreased the protein levels of vimentin in SW480 cells (Fig. 3B).

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Figure 3. Knockdown of PFTK1 suppresses EMT in colon cancer cells. HT29 and SW480 cells were infected with siRNA-PFTK1 or mock for 24 h. (A) The protein levels of vimentin and E-cadherin were determined by Western blot in HT29 cells, and relative protein levels were quantified using Image-Pro Plus 6.0 software. (B) The protein levels of vimentin and E-cadherin were determined by Western blot in SW480 cells, and relative protein levels were quantified using Image-Pro Plus 6.0 software. All the experiments were repeated at least three times. * $p < 0.05$ versus mock.

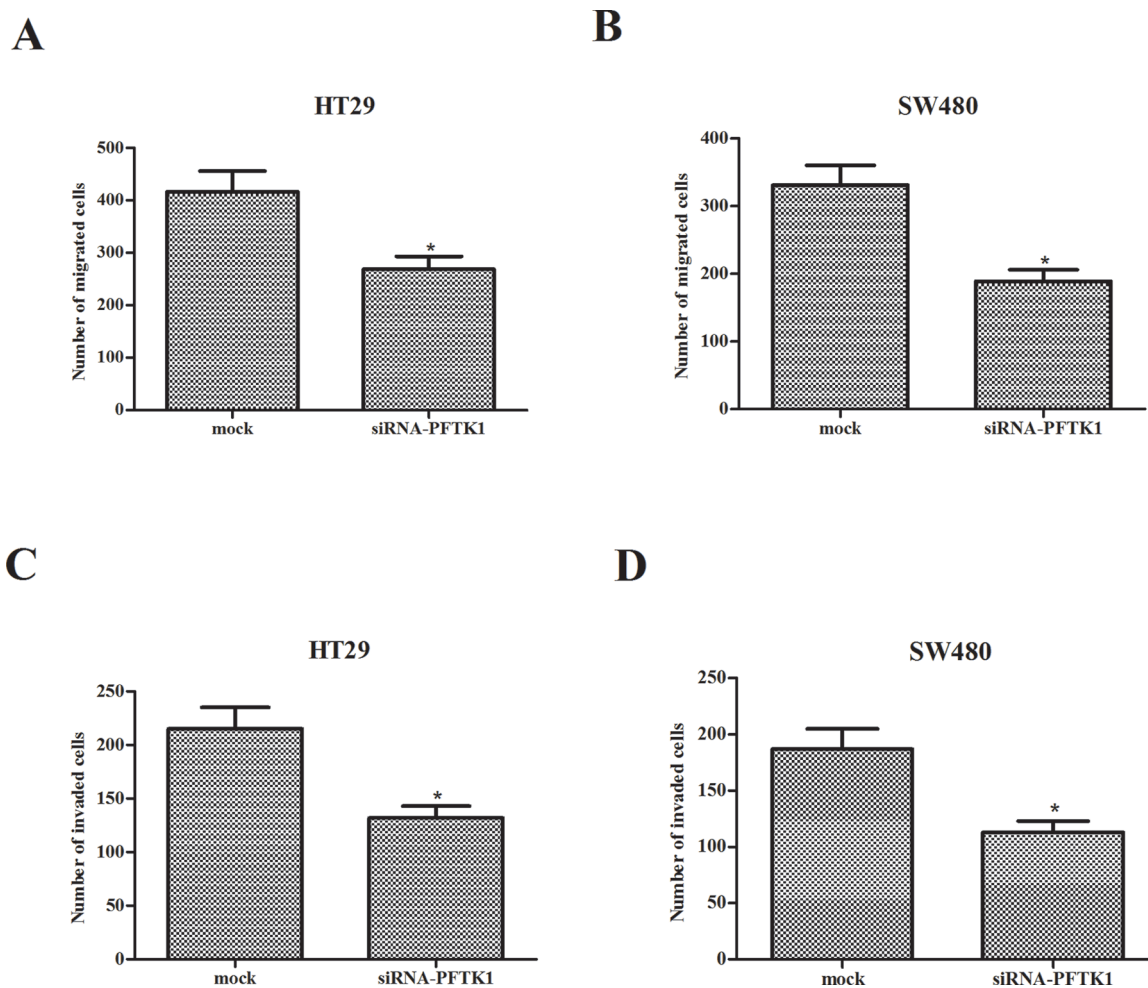


Figure 4. Knockdown of PFTK1 inhibits colon cancer cell migration and invasion. HT29 and SW480 cells were infected with siRNA-PFTK1 or mock for 24 h. (A, B) Cell migration of HT29 and SW480 cells transfected with siRNA-PFTK1 was measured by Transwell migration assay. (C, D) Cell invasion of HT29 and SW480 cells transfected with siRNA-PFTK1 was assessed by the Matrigel invasion chamber. All the experiments were repeated at least three times. * $p < 0.05$ versus mock.

Knockdown of PFTK1 Inhibits Colon Cancer Cell Migration and Invasion

To examine the effect of PFTK1 on colon cancer cell migration and invasion, we performed Transwell migration assay and Boyden chamber invasion assay. Transwell migration assay showed that the number of migrated cells was significantly smaller in the siRNA-PFTK1-transfected groups compared to the mock groups (Fig. 4A and B). In addition, Boyden chamber invasion assays demonstrated that downregulation of PFTK1 also decreased invasive capacity of HT29 and SW480 cells, respectively (Fig. 4C and D).

Knockdown of PFTK1 Inhibits the Sonic Hedgehog Signaling Pathway in Colon Cancer Cells

The Sonic hedgehog (Shh) signaling pathway plays a critical role in tumorigenesis by regulating cell

proliferation, cell survival, cell migration, and invasion. To further investigate the underlying mechanism of siRNA-PFTK1-inhibited colon cancer cell proliferation and invasion, we investigated the effect of PFTK1 on Shh pathway activation in colon cancer cells. As shown in Figure 5, knockdown of PFTK1 obviously inhibited the protein expression of Shh as well as Smo, Ptc, and Gli-1 in HT29 cells when compared with the mock group.

DISCUSSION

Numerous tumor suppressor genes and oncogenes have been identified in colon cancer, and further studies of these gene alterations and functions will assist in revealing the molecular mechanisms of colon cancer progression. In this study, we found that PFTK1 was highly expressed in human colon cancer cell lines. In addition, we found that knockdown of PFTK1 significantly inhibits the proliferation and

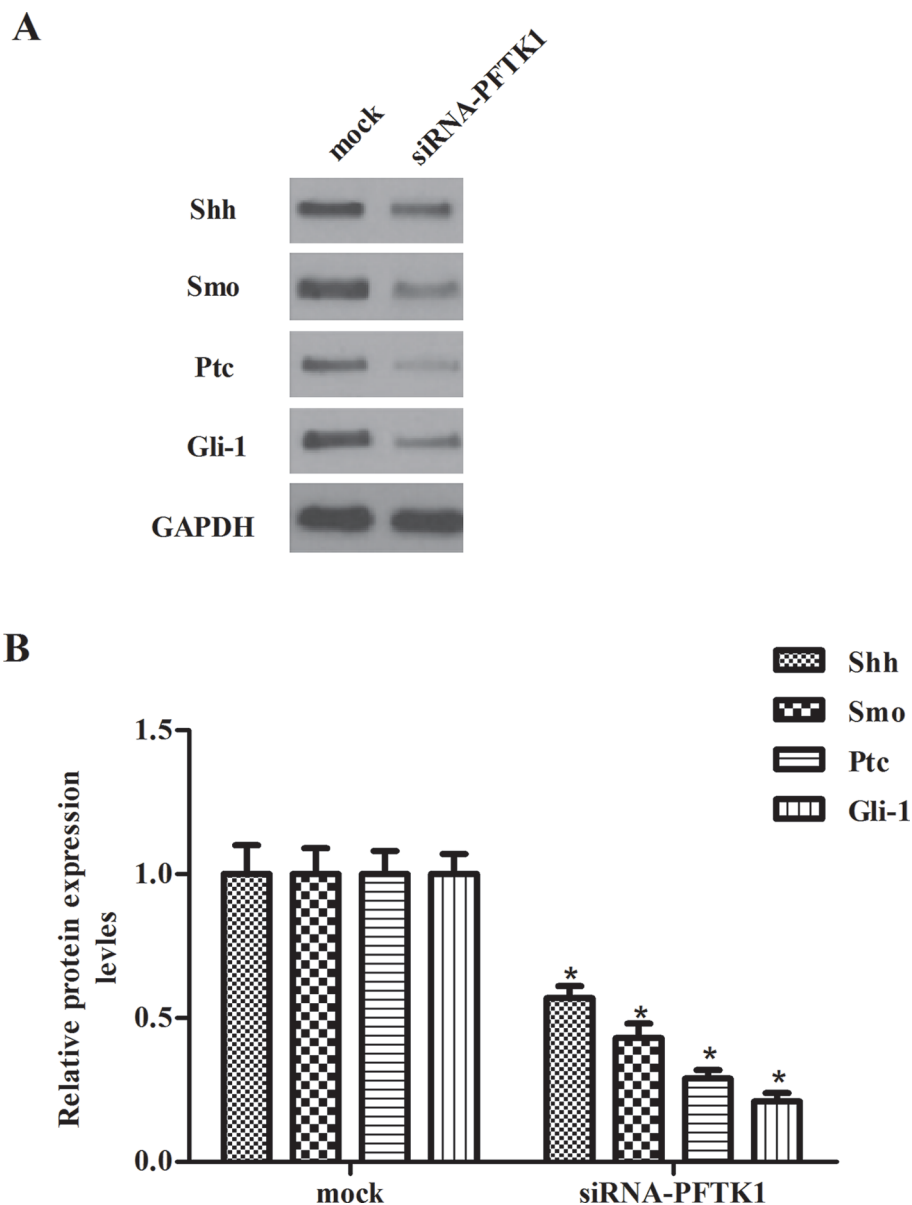


Figure 5. Knockdown of PFTK1 inhibits the Sonic hedgehog signaling pathway in colon cancer cells. HT29 cells were infected with siRNA-PFTK1 or mock for 24 h. (A) The protein levels of Shh, Smo, Ptc, and Gli-1 were determined by Western blot. (B) The relative protein levels were quantified using Image-Pro Plus 6.0 software. All the experiments were repeated at least three times. * $p < 0.05$ versus mock.

EMT program, as well as cell migration and invasion in colon cancer cells. Furthermore, knockdown of PFTK1 obviously inhibits the protein expression of Shh as well as Smo, Ptc, and Gli-1 in colon cancer cells.

PFTK1 was shown to play a critical role in cancer development and progression. Gu et al. showed that PFTK1 is overexpressed in breast cancer patients, and knockdown of PFTK1 attenuated breast cancer cell proliferation (10). Yang et al. confirmed that PFTK1 overexpression promoted proliferation in gastric cancer cells

(11). In agreement with these results, we observed that PFTK1 is highly expressed in human colon cancer cell lines, and knockdown of PFTK1 inhibits colon cancer cell proliferation. These data suggest that PFTK1 may be an oncogene in the progression of colon cancer.

EMT plays a critical role in driving colon cancer invasion and metastasis (3,12,13). During the EMT procedure, the actin cytoskeleton is dramatically reorganized, and cells acquire increased cell-matrix contacts, leading to dissociation from surrounding cells and enhanced

migratory and invasive properties (14). Previous studies demonstrated that the loss or reduction of E-cadherin contributes to the development and progression of colon cancer (15). Herein, we observed that knockdown of PFTK1 greatly increased the expression level of E-cadherin, but decreased the expression level of vimentin. Moreover, we found that knockdown of PFTK1 inhibits cell migration and invasion in colon cancer cells. These results suggest that PFTK1 might be an important contributor to EMT progression, thus facilitating the migration and invasion of colon cancer cells.

The Shh signaling pathway plays an important role in the development and progression of colon cancer (16–18). Shh is the main component of this pathway. Ptc1, a receptor of Hh signaling pathway, can suppress the transmembrane protein smoothed (Smo). Increasing evidence has reported that the main mechanism of the Hh signaling pathway in colon cancer cells was to promote invasion and the EMT process. For example, Coultas et al. reported that Gli-1 upregulates the expression of Snail, which is a repressor of the gene expression of E-cadherin often implicated in the metastasis of colorectal cancer (19), and knockdown of Gli-1 using siRNA reduced proliferation and induced apoptosis in primary cultures of human colon cancers and liver metastasis (20). Another study reported that mRNA and protein levels of Ptc1 were inversely correlated with the metastatic potential of colorectal cancer cells (21). In addition, several studies showed that blocking this pathway significantly inhibited metastasis of tumor cells in vivo (22,23). In this study, we found that knockdown of PFTK1 obviously inhibited the protein expression of Shh as well as Smo, Ptc, and Gli-1 in colon cancer cells. These results suggest that knockdown of PFTK1 inhibits colon cancer cell migration/invasion and EMT through suppressing the Shh signaling pathway.

In conclusion, these results demonstrate that PFTK1 may play an important role in colon cancer cell proliferation, invasion, and the EMT progress. Thus, PFTK1 may be a potential therapeutic target for the treatment of colon cancer.

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