High Rab11-FIP4 expression predicts poor prognosis and exhibits tumor promotion in pancreatic cancer

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Abstract. Some studies have demonstrated that Rab11-family interacting proteins (Rab11-FIPs) are connected with the tumorigenesis, and they may act as tumor promoters in some cancers. The clinicopathological significance of Rab11-family interacting protein 4 (Rab11-FIP4) expression and its possible effects on pancreatic cancer (PC) are still undiscovered. In this study, Rab11-FIP4 protein expression level in 60 PC specimens and pair-matched non-cancerous samples were detected by immunohistochemistry analysis. The results were analysed and compared with each patients' clinical data. Rab11-FIP4 expression in PC tissues increased significantly more than that of adjacent non-cancerous tissues (P=0.0001). Overexpression of Rab11-FIP4 in the PC tissues was significantly related to tumor size (P=0.0001), histological grade (P=0.028), metastasis (P=0.001) and TNM stage (P=0.004) but not with age (P=0.832), gender (P=0.228) or tumor site (P=0.875). Kaplan-Meier survival analysis showed that overexpression of Rab11-FIP4 was significantly related to overall survival time (P=0.0036). In addition, Rab11-FIP4 in PANC-1 pancreatic cancer cells were successfully knocked-out using the CRISPR/Cas9 system. Rab11-FIP4 knockout in PANC-1 cells inhibited cell growth, invasion and metastasis, and arrested cell cycle progression, but did not alter apoptosis. Our findings suggest that overexpression of Rab11-FIP4 predicts poor clinical outcomes for pancreatic cancer and contributes to pancreatic tumor progression.

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Introduction

Pancreatic cancer (PC) is one of the most common causes of cancer mortality in the United States (1). Because of the aggressive growth and metastatic tendencies of PC, PC patients usually enter advanced stage when diagnosed. Therapeutic methods, including surgery and medical interventions, were mainly unsuccessful, and the prognosis was often not optimistic (1,2). Previous studies have illuminated multiple potential pathogenicity mechanisms and risk factors for PC (3); however, the recurrent or metastatic molecular mechanism of PC is still unknown. As a member of the small GTPase family, Rab11 has a significant influence on vesicular trafficking, especially translocation of proteins from the trans-Golgi network to the plasma membrane and recycling of membrane protein, as well as cytokinesis (4-6). The C-termini of Rab11-family interacting proteins (Rab11-FIPs) possess a highly conserved short motif, called the Rab11-binding domain, that interacts strongly with Rab11-GTP (7). Rab11-FIPs have three main functions: recycling of cargo to the membrane surface, transport of membrane to the cleavage furrow or midbody during cell division and linking Rab11 to molecular motor proteins (8). It is different from other Rab11-FIPs in that the N-terminus of Rab11-FIP4 possesses an EF-hand calcium-binding motif (8,9). Rab11-FIP4, as a downstream effector of Rab11, plays an important role in the membrane trafficking in association with cytokinesis (10). The proliferation and differentiation of retinal progenitor cells is regulated by Rab11-FIP4 in the process of mouse and zebrafish retinal development (11,12). Hypoxia increases tumor cell invasion by modulating Rab11 (13). The invasion and metastasis potential of hepatocellular carcinoma (HCC) were significantly promoted by hypoxia-induced Rab11-FIP4. The level of Rab11-FIP4 was

overexpressed in HCC and contributed to an unfavourable clinical outcome in HCC patients (14). Hypoxia is significantly correlated with tumor growth, metastasis and poor clinical outcome in PC (15,16). However, at present, the underlying effect of Rab11-FIP4 on PC progression remains unknown.

In this study, we investigate the expression level of Rab11-FIP4 in PC tissues and the relationship with clinicopathological features of PC. Furthermore, whether Rab11-FIP4 plays critical roles in PC progression were also studied.

Materials and methods

Patients and specimens. Sixty pairs of pancreatic tumor specimens and 60 corresponding para-carcinoma tissue samples were acquired from the Second Affiliated Hospital and the First Affiliated Hospital of Wenzhou Medical University, China. Tumor grade and stage were on the basis of the American Joint Committee on Cancer/International Union Against Cancer staging manual (2009). Patient clinical information and tumor characteristics are summarized in Table I. All patients were followed-up 22-60 months (mean, 48.2 months) after the surgery. All PC tissue specimens were acquired using protocols approved by the Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University, and all patients signed a written informed consent.

Immunohistochemistry analysis. The detection of Rab11-FIP4 expression in pancreatic cancer specimens followed a standard immunohistochemistry protocol. Ordinarily, to retrieve the antigen, the deparaffinized and rehydrated slides were placed into boiling ethylenediamine tetraacetic acid buffer. Then, the samples were incubated in 3% H_2O_2 (10 min, room temperature) to eliminate the activity of endogenous peroxidases. After 5% goat serum blocking, the slides were applied with anti-Rab11-FIP4 antibody (Santa Cruz Biotechnology, CA, USA) with a dilution of 1:100 at 4˚C overnight in a humidified chamber. All the sections were incubated with a biotinylated secondary antibody for 2 h, followed by incubation with a streptavidin-horseradish-peroxidase complex. Sequentially, the sections were stained with 3,3'-diaminobenzidine (DAB) and then counter-stained with Mayer's hematoxylin. The Rab11-FIP4 expression in the pancreatic tumor samples was independently assessed by two clinical pathologists. Immunohistochemical evaluation of the Rab11-FIP4 protein was performed according to previously described methods, taking into account the extent (graded from 0 to 4) and intensity (0-3) of immunopositivity (17,18). We multiplied the intensity score with positive extent to define the final score, and a final score range was 0-12. Final scores of ≥4 were considered as high expression, and scores of <4 were considered as low expression.

Construction. According to the Rab11-FIP4 gene sequence (forward, 5'-GAGAATGACAGCCTGACCAAT-3'), three single-guide RNAs (sgRNAs) were constructed on a lentiviral backbone: sgRNA-1, forward, 5'-TGTCGGGAGTCCTGCCGAGA-3'; sgRNA-2, forward, 5'-CTGATGGCGAGCTCATCCCC-3'; sgRNA-3, forward, 5'-AGCCCGACTGAAAAACCTGA-3', and a negative control (NC) sgRNA (5'-CGCTCGCGGCCCGTTCAA-3') duplex was chemically synthesized. Then, the corresponding Rab11-FIP4-sgRNA oligos were synthesized (Rab11-FIP4-sgRNA-1, forward, 5'-caccgTGTCGGGAGTCCTGCCGAGA-3'; Rab11-FIP4-sgRNA-2, forward, 5'-caccgCTGATGGCGAGCTCATCCCC-3'; Rab11-FIP4-sgRNA-3, forward, 5'-caccgAGCCCGACTGAAAAACCTGA-3'). The resulting Rab11-FIP4-sgRNAs for the target site were cloned into the vector GV371 (GeneChem Co. Ltd. Shanghai, China), which contained the EGFP reporter, to produce recombinant plasmid GV371-Rab11-FIP4-sgRNA. The other plasmid vector, Lenti-CAS9-puro, was purchased from GeneChem Co. Ltd. Virus packaging was completed in 293T cells after the co-transfection of the GV371-Rab11-FIP4-sgRNA plasmid or the Lenti-CAS9-puro plasmid with the packaging plasmid.

Cell culture and transfection. The human PC cell lines, CFPAC-1, PANC-1 and AsPC-1, were acquired from the Shanghai Cell Bank of the Chinese Academy of Sciences. All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (Mediatech, VA, USA) and incubated in a 37˚C chamber with 5% CO_2.

We used the CRISPR/Cas9 (19-22) system to construct a double vector lentiviral vector. First, the Lenti-CAS9 lentivirus transfected PANC-1 cells. After 72 h, the PANC-1 cells that stably expressed Cas9 were selected by puromycin (Clontech, San Francisco, CA, USA). Then, the stably expressing Cas9-PANC-1 cells were transfected with the sgRNA lentivirus. A fluorescent microscope (Olympus, Tokyo, Japan) was used to detect EGFP expression. When the lentiviral transfection efficiency was greater than 80%, the cells were harvested for the experiment.

Detection of introduced mutations in genomic DNA. The Cruiser nuclease digestion assay was conducted to detect introduced mutations in the genomic DNA using the Knockout and Mutation Detection kit (GeneChem Co. Ltd.). The cells were harvested 7 days after transfection, and DNA was extracted using a Genomic DNA extraction kit (Tiangen, Beijing, China) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of the region containing the target site was carried out using the following primers: sgRNA-1, forward, 5'-TGTCGGGAGTCCTGCCGAGA-3'; sgRNA-2, forward, 5'-CTGATGGCGAGCTCATCCCC-3'; and sgRNA-3, forward, 5'-AGCCCGACTGAAAAACCTGA-3'; then, the PCR products were annealed. The annealed PCR products were naturally cooled below 40˚C at room temperature. Then, the PCR products (3 μl) were examined by electrophoresis. Lastly, in a sterile PCR tube, the reaction solution containing 3 μl PCR products, 2 μl detecase buffer, 1 μl detecase and 4 μl ddH_2O were combined. After a nuclease digestion reaction lasting 20 min at 45˚C, 2 μl of stop buffer was added to the reaction solution. Subsequently, all the reaction solutions were detected by 2% agarose gel electrophoresis.

MTT assay. To measure the cell proliferation after transfection with Rab11-FIP4, the MTT assay was performed. The cells ($2x10^3$ cells/well) were seeded into 96-well culture plates (Corning, CA, USA). Then, 20 μl of MTT reagent (5 mg/ml;

Table I. The relationship between Rab11-FIP4 expression and clinicopathological features.

Clinicopathological feature	Parameter	Rab11-FIP4		P-value
		Low	High	
Age	≥60	20	16	0.832
	<60	14	10	
Gender	Male	21	12	0.228
	Female	13	14	
Tumor site	Head	19	14	0.875
	Body, tail	15	12	
Size	>3 cm	7	19	0.0001
	≤3 cm	27	7	
Histologic grade	Well to moderate	24	11	0.028
	Poor	10	15	
Metastasis	Yes	10	19	0.001
	No	24	7	
TNM stage	0-IIa	22	7	0.004
	IIb-IV	12	19	
Survival	Mean (months)	28.5	16.4	0.0036

Genview, FL, USA) was added after 24, 48, 72, 96 and 120 h. After 4-h incubation at 37˚C, the medium was replaced with 100 µl of DMSO (Shanghai Shiyi Chemical Reagent Co. Ltd., Shanghai, China), and the plate was rotated for 2-5 min at room temperature. The spectrometric absorbance was measured at 490 nm with a microplate reader (Tecan Infinite, Mannedorf, Switzerland).

Apoptosis assay. Seven days after transfection, apoptosis of transfected PANC-1 cells was detected by Annexin V-APC (eBioscience, CA, USA) single staining. In brief, these cells were washed two times with complete culture medium and were incubated in 200 µl binding buffer and 10 µl Annexin V-APC at room temperature for 15 min in the dark. Then, the stained cells were analysed by flow cytometry (Millipore, MA, USA).

Cell cycle analysis. Flow cytometry was used to detect the cell cycle distribution. In brief, the cells were washed and resuspended in staining buffer containing 10 µg/ml propidium iodide (PI) and 25 µg/ml RNase. The cell cycle was analysed by the Guava easyCyte HT flow cytometer (Millipore) following staining. Histograms were used to stand for the proportion of cells in phase G1, S and G2/M.

Migration and invasion assay. Migration and invasion experiments were performed with PC cells using a 24-well Transwell plate (Corning). For the migration assay, stably transfected cells (PANC-1) in 100 µl of serum-free DMEM were plated in the upper chamber. Then, 600 µl of DMEM supplemented with 30% FBS was placed in the lower chamber. After the cells were incubated at 37˚C for 24 h, the cells that could not migrate were removed from the upper surface of the membrane with a cotton swab. The cells that had passed through the membranes were stained with 0.1% rystal violet for 10 min. The migrating cells were viewed under an inverted microscope (Olympus, Tokyo, Japan), and nine random fields (magnification, x200) were photographed and counted.

For the invasion assay, stably transfected cells (PANC-1) in 500 µl of serum-free DMEM were plated in the upper chamber of Matrigel-precoated (Becton-Dickinson Co., NJ, USA) Transwell plates. Then, 750 µl of DMEM supplemented with 30% FBS was placed in the lower chamber. After the cells were incubated at 37˚C for 40 h, the non-invading cells were removed from the upper surface of the membrane, and the cells on the lower surface of the insert were stained and counted as the migration assay. The experiment was performed three times.

Real-time PCR. TRIzol reagent (Pu Fei, Shanghai, China) was used to extract total RNA from cultured PC cells following the kit instructions. Reverse transcription was performed using the M-MLV Reverse Transcription kit (Promega, WI, USA) according to the manufacturer's instructions. Real-time PCR (RT-PCR) was subsequently performed with the SYBR Premix Ex Taq (Takara, Dalian, China). The PCR reaction conditions were as follows: 95˚C for 30 sec followed by 40 cycles of 95˚C for 10 sec and 60˚C for 30 sec. The sequences of the primers used were as follows: Rab11-FIP4, forward, 5'-GAGAATGACAGCCTGACCAAT-3'; reverse, 5'-GCTCTGTATTTTCTTCCTCCAAC-3'. GAPDH, forward, 5'-TGACTTCAACAGCGACACCCA-3'; reverse, 5'-CACCCTGTTGCTGTAGCCAAA-3'. The mRNA relative expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis. IBM SPSS Statistics software program version 22.0 (IBM Corp., NY, USA) was used to process the data. Data are presented as the means ± SEM of values from three independent experiments. Statistical significance among different groups was analysed by one-way analysis of variance (ANOVA). The correlation between Rab11-FIP4 and the clinicopathological features were assessed by χ^2 test and Fisher's exact test. The Kaplan-Meier method with the log-rank test or Cox regression method was used to evaluate overall survival. A P-value <0.05 was considered statistically significant.

Results

Overexpression of Rab11-FIP4 in PC is related to the clinicopathologic features of the PC patients. The immunohistochemical results revealed that the Rab11-FIP4 protein was highly expressed in 26 (26/60, 43.3%) PC patients (Fig. 1A) and only in 5 (5/60, 8.3%) adjacent non-cancerous pancreatic tissues (P=0.0001). Moreover, the Rab11-FIP4 protein expression in most of the adjacent non-cancerous pancreatic tissues was negative (Fig. 1B). Interestingly, the staining density was related to histological grade, and high Rab11-FIP4 expression was more often observed in poorly differentiated pancreatic carcinoma tissues (Fig. 1C-E). Rab11-FIP4 overexpression was related to tumor size, histological grade, metastasis and TNM stage, but not with age, gender or tumor site in the 60 pancreatic tumor specimens (Table I).

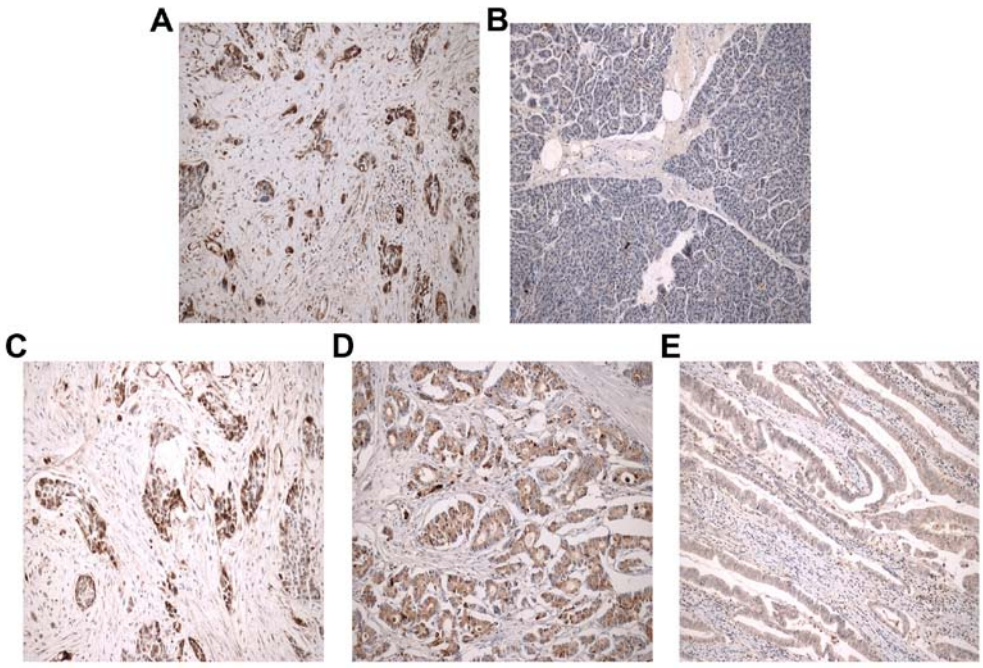


Figure 1. Rab11-FIP4 expression level in pancreatic cancer (PC) and corresponding non-tumor normal tissues detected by immunohistochemistry. (A) Rab11-FIP4 protein was highly expressed in PC cells. (B) Rab11-FIP4 protein expression was negative in corresponding non-tumor normal tissues. The staining intensity of Rab11-FIP4 protein by immunohistochemistry in poorly differentiated (C), moderately differentiated (D) and well-differentiated (E) PC cells (x200).

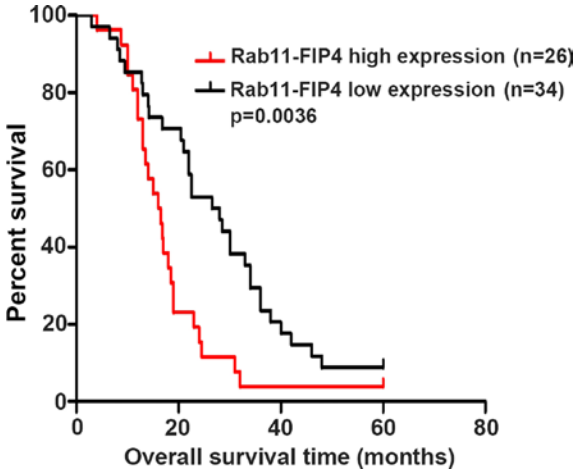


Figure 2. Expression of Rab11-FIP4 in PC patients and its correlation with overall survival. Kaplan-Meier survival analysis showed that high Rab11-FIP4 expression group has much shorter mean overall survival (OS) time than that of low Rab11-FIP4 expression group (16.4 vs. 28.5 months).

Relationship between Rab11-FIP4 expression and prognosis of PC patients. We studied whether Rab11-FIP4 overexpression was related to the overall survival of PC patients. The sixty PC patients were divided into two groups according to the immunohistochemical results. The overall survival time of high Rab11-FIP4 expression group was (16.4±5.9) months, while it was (28.5±12.7) months in low Rab11-FIP4 expression group. The results indicated that patients with low Rab11-FIP4 expression had much longer OS times (P=0.0036) (Fig. 2).

Rab11-FIP4 knockout in PANC-1 cells using the CRISPR/Cas9 system. Rab11-FIP4 expression was analysed in several PC cell lines using RT-PCR. The relative Rab11-FIP4 expression level is shown in Fig. 3A. The cell line PANC-1, moderately expressed Rab11-FIP4, exhibiting a relative expression level of 15.368 ± 0.132 (Fig. 3A). Therefore, PANC-1 cells were selected for the subsequent cellular function test of Rab11-FIP4 in PC.

To verify whether the three sgRNAs contained the positions corresponding to the loci of single nucleotide polymorphisms (SNPs), the sgRNA fragments were amplified by PCR, and the PCR products were sequenced. As shown in Fig. 3B, the sequencing results showed that the three sgRNAs had no SNP loci. The corresponding positions of sgRNA-1 and sgRNA-2 were very close, so their PCR amplification primers were the same.

We used the CRISPR/Cas9 genome-editing technique, which has been reported to efficiently knock out genes in various organisms (22). After GV371-Rab11FIP4-sgRNA Lentivirus efficiently transfected the stably expressed Cas9-PANC-1 cells, Cruiser-nuclease digestion assay was used to detect sgRNA knockout activity. As shown in Fig. 3C, compared to the negative control group, the cutting strip appeared in the expected position in the experimental groups (KO1, KO2 and KO3). This result illustrated that the sgRNA has efficient knockout activity. The knockout activity of KO1 was more efficient than KO2 and KO3, so KO1 was selected for functional experiments. The result also showed that the mutation was successfully induced at the Rab11-FIP4 gene target locus. Therefore, Rab11-FIP4 was efficiently knocked out by the CRISPR/CAS9 double vector lentiviral system (Fig. 3D and E).

Effect of Rab11-FIP4 knockout on PANC-1 cell proliferation. The effect of Rab11-FIP4 on the growth ability of PC

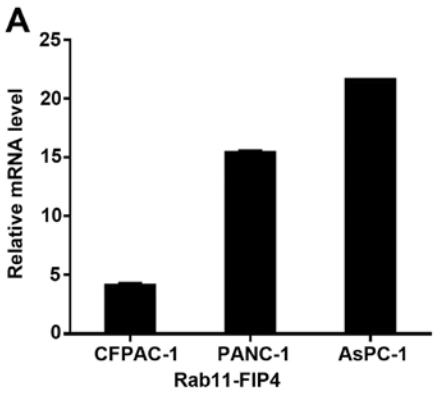
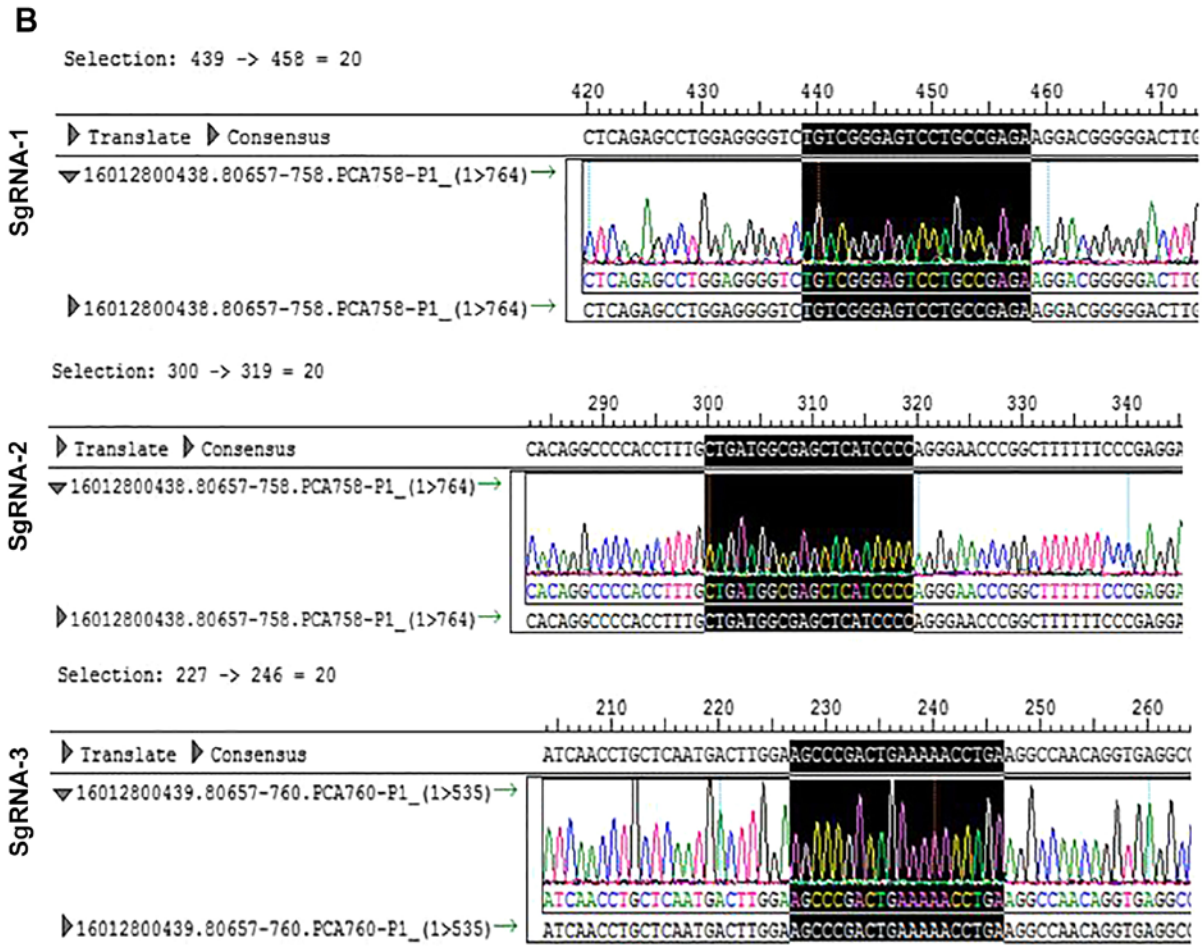
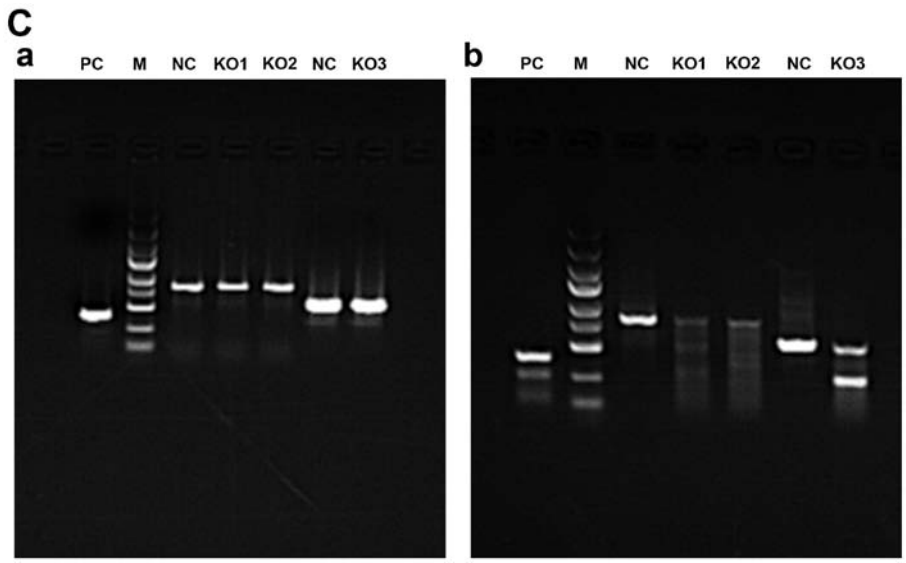


Figure 3. Rab11-FIP4 knockout in PC cells. (A) RT-PCR analysis of Rab11-FIP4 mRNA expression in PC cell lines. Rab11-FIP4 was highly expressed in PC cells. (B) The sgRNA detection sequences. (C) Cruiser nuclease digestion analysis confirming the activity of the sgRNA on its target site in PANC-1 cells. (a) PCR electrophoretogram before nuclease digestion. (b) PCR electrophoretogram after nuclease digestion.





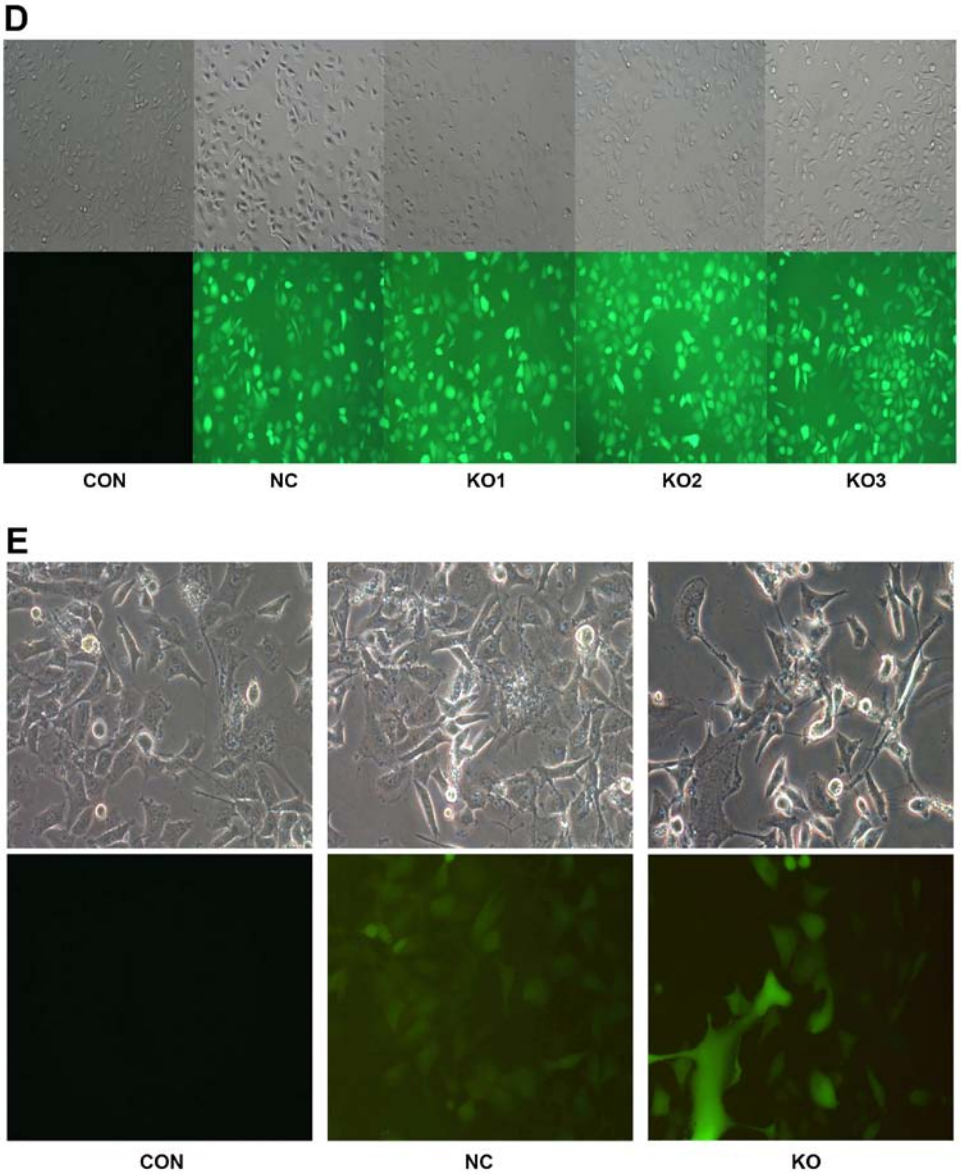


Figure 3. Continued. (D) The transfection of each lentiviral sgRNA vector (CON, NC, KO1, KO2 and KO3) in PANC-1 cells as evaluated by fluorescence microscopy. (E) The transfection and knockout efficiency were estimated 3 days after infection with the CRISPR/Cas9 double vector lentivirus. Enhanced green fluorescent protein (EGFP) expression in infected cells was observed by light and fluorescence microscopy, respectively. Light micrograph (up). Fluorescent micrograph (down) (x200). CON, no infection group. NC, negative control group. KO, Rab11-FIP4 knockout group.

cells was assessed by MTT assay. As shown in Fig. 4, the EGFP-expressing transfected cells were counted once a day for 5 days. The proliferation rates of PANC-1 cells markedly decreased following Rab11-FIP4 knockout on days 4 and 5 ($P<0.05$).

Effect of Rab11-FIP4 knockout on PANC-1 apoptosis. In order to study the effect of Rab11-FIP4 knockout on PANC-1 apoptosis, we used flow cytometry to identify the apoptosis rate. As shown in Fig. 5, the percentage of apoptosis was 4.73±0.82% in the negative control group (NC) cells and 2.55±0.20% in knockout group (KO) cells ($P<0.05$). Because the apoptosis rate of each group was <5%, the result showed that the cells were not undergoing apoptosis.

Effect of Rab11-FIP4 knockout on the PANC-1 cell cycle. PANC-1 cells were transfected with the CRISPR/Cas9 double

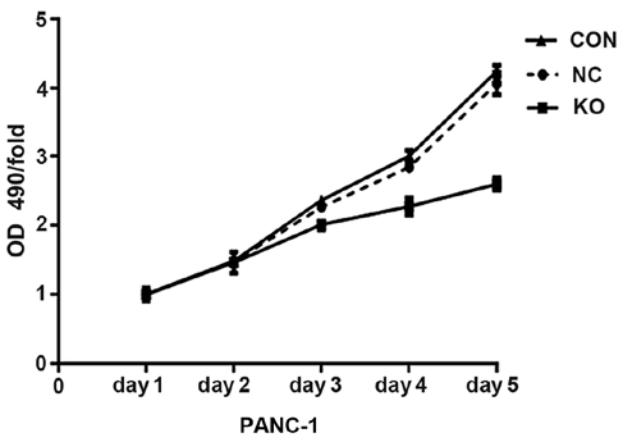


Figure 4. MTT assay indicated that Rab11-FIP4 knockout inhibited the proliferation of PANC-1 cells ($P<0.05$). CON, no infection group. NC, negative control group. KO, Rab11-FIP4 knockout group.

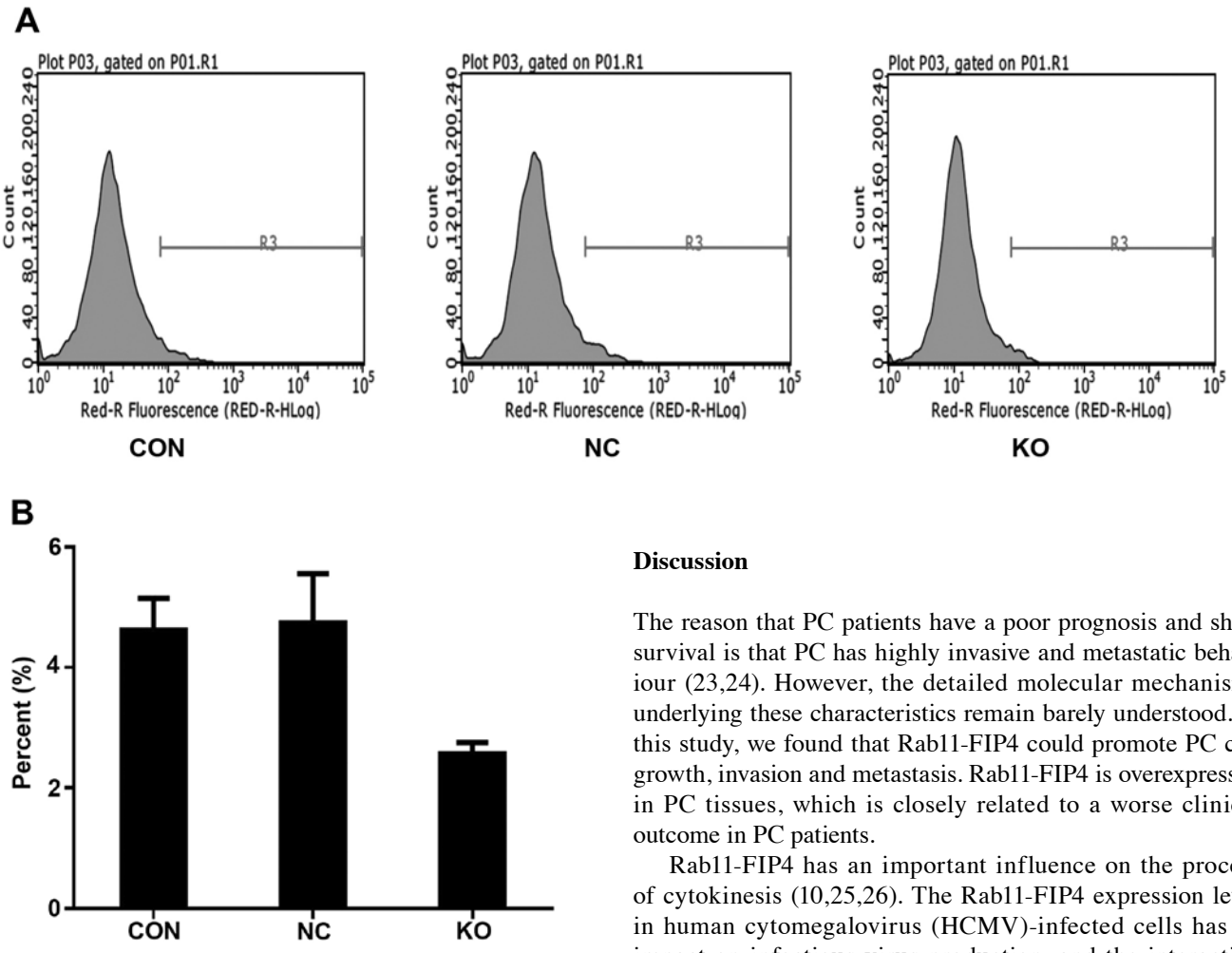


Figure 5. Effect of Rab11-FIP4 knockout on PANC-1 apoptosis (A and B) Rab11-FIP4 knockout did not significantly alter the apoptosis of PANC-1 cells. CON, no infection group. NC, negative control group. KO, Rab11-FIP4 knockout group.

vector lentivirus for 7 days, and cell cycle distribution was assessed by flow cytometry. There were significant differences in cell cycle progression, including G1, S and G2/M phases, between the Rab11-FIP4 KO and NC. As shown in Fig. 6, the Rab11-FIP4 knockout significantly reduced the S stage fraction (KO 42.51%, NC 40.38%; P=0.0073) and G2/M stage fraction (KO 21.49%, NC 18.52; P=0.0002) while increasing the G1 stage fraction (KO 36%, NC 41.1%; P=0.0001). These data indicate that Rab11-FIP4 knockout suppressed PANC-1 cell cycle progression.

Effect of Rab11-FIP4 knockout on PANC-1 cell migration and invasion. To study the role of Rab11-FIP4 in PANC-1 cell migration and invasion, we stably knocked out Rab11-FIP4 in PANC-1 cells using CRISPR/CAS9 double vector lentiviral system. Transwell assays and Matrigel invasion assays were performed to detect the migratory and invasive capacities of PANC-1 cells. As shown in Fig. 7, the Rab11-FIP4 knockout group had markedly fewer migrating and invading cells than the parental control group. These results suggest that knockout of Rab11-FIP4 remarkably inhibited the migratory and invasive ability of PANC-1 cells.

Discussion

The reason that PC patients have a poor prognosis and short survival is that PC has highly invasive and metastatic behaviour (23,24). However, the detailed molecular mechanisms underlying these characteristics remain barely understood. In this study, we found that Rab11-FIP4 could promote PC cell growth, invasion and metastasis. Rab11-FIP4 is overexpressed in PC tissues, which is closely related to a worse clinical outcome in PC patients.

Rab11-FIP4 has an important influence on the process of cytokinesis (10,25,26). The Rab11-FIP4 expression level in human cytomegalovirus (HCMV)-infected cells has an impact on infectious virus production, and the interaction between Rab11-FIP4 and Rab11 regulates vesicular transport during cytokinesis (27). Recent studies have indicated that Rab11-FIP4 acts as a Rab11 effector in HeLa cells and performs cellular functions other than transferrin recycling (28). Rab11-FIP4 expressed abundantly in the mouse and zebrafish nerve tissues and promoted the growth of retinal progenitors (11,12). Rab11-FIP4 is a member of the family of the Rab11-FIPs, which is composed of six members (7). Rab11-FIPs are likely to be related to tumorigenesis, and they may act as tumor promoters in some cancers. Rab11-FIPs are closely associated with the occurrence and metastasis of HCC, gastric cancer, breast cancer and colorectal cancer. The expression of Rab11-FIPs is increased significantly in some tumor tissues (12,29-32). For instance, Rab11-FIP2 overexpression enhanced gastric cancer cell ability of invasion and metastasis (32). The studies on the relationship between Rab11-FIP4 and malignant tumors are not extensive. The latest study demonstrated that Rab11-FIP4, functioning as regulatory factor, promoted hepatocellular carcinoma cell invasion and metastasis. Hypoxia upregulated Rab11-FIP4 expression levels in HCC cells. Also, the study revealed that the high expression of Rab11-FIP4 in hepatocellular cancer tissues had a significant correlation with lower survival rates (14). The results of our study are consistent with the above.

In our study, we first observed Rab11-FIP4 expression in PC tissues. Immunohistochemistry analysis demonstrated that the expression of Rab11-FIP4 in PC tissues was higher than adjacent non-tumorous tissues. Clinical and pathological data

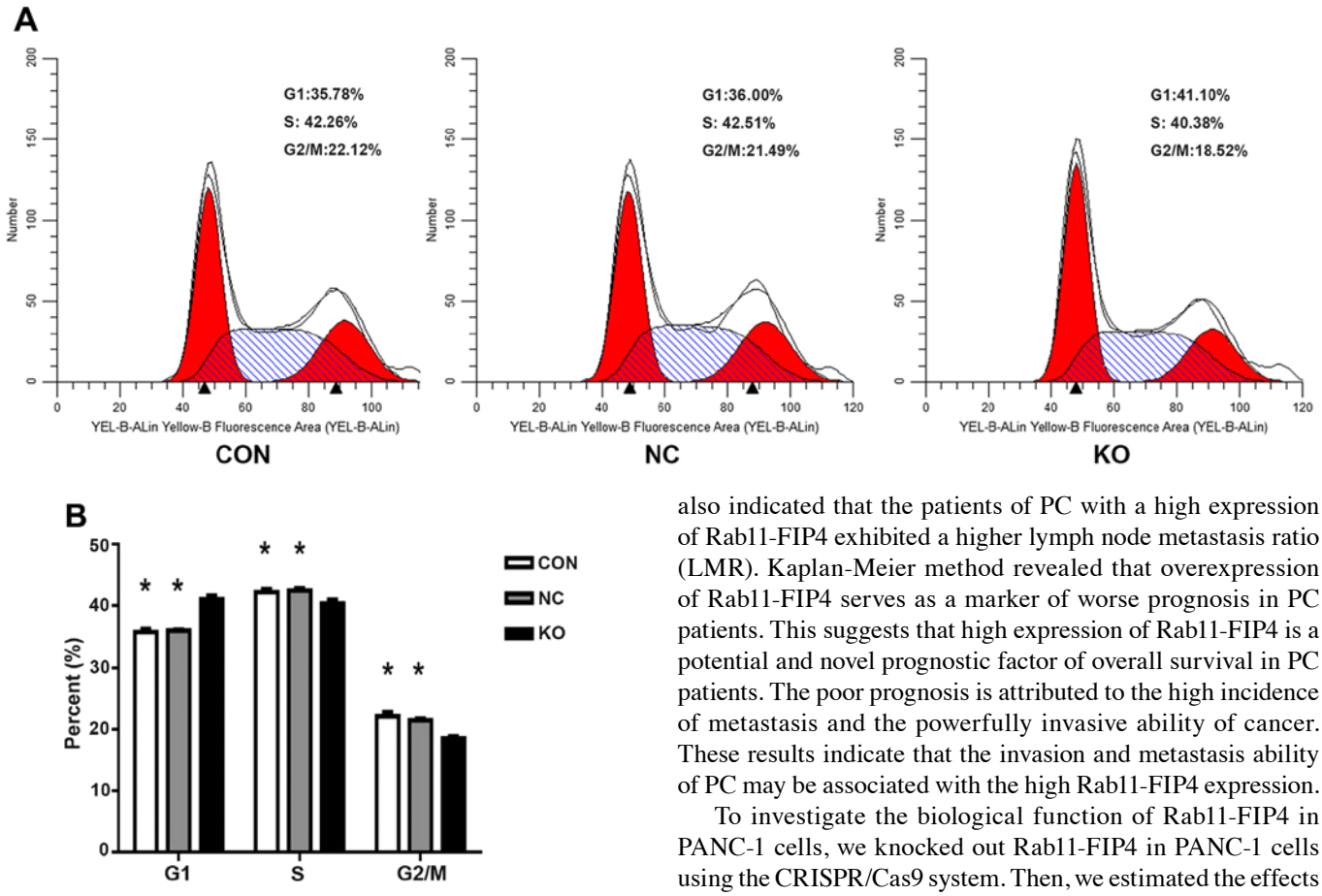


Figure 6. Effect of Rab11-FIP4 knockout on the PANC-1 cell cycle. (A) Cell cycle analysis by flow cytometry. (B) Rab11-FIP4 knockout arrested PANC-1 cells in G1 phase (P<0.05). CON, no infection group. NC, negative control group. KO, Rab11-FIP4 knockout group. *P-value, compared with the KO, P<0.05.

also indicated that the patients of PC with a high expression of Rab11-FIP4 exhibited a higher lymph node metastasis ratio (LMR). Kaplan-Meier method revealed that overexpression of Rab11-FIP4 serves as a marker of worse prognosis in PC patients. This suggests that high expression of Rab11-FIP4 is a potential and novel prognostic factor of overall survival in PC patients. The poor prognosis is attributed to the high incidence of metastasis and the powerfully invasive ability of cancer. These results indicate that the invasion and metastasis ability of PC may be associated with the high Rab11-FIP4 expression.

To investigate the biological function of Rab11-FIP4 in PANC-1 cells, we knocked out Rab11-FIP4 in PANC-1 cells using the CRISPR/Cas9 system. Then, we estimated the effects of Rab11-FIP4 on PANC-1 cell proliferation and discovered that Rab11-FIP4 knockout had a significant effect on PANC-1 cell proliferation. We found that deficiency of Rab11-FIP4 in PANC-1 cells did not significantly alter apoptosis, suggesting

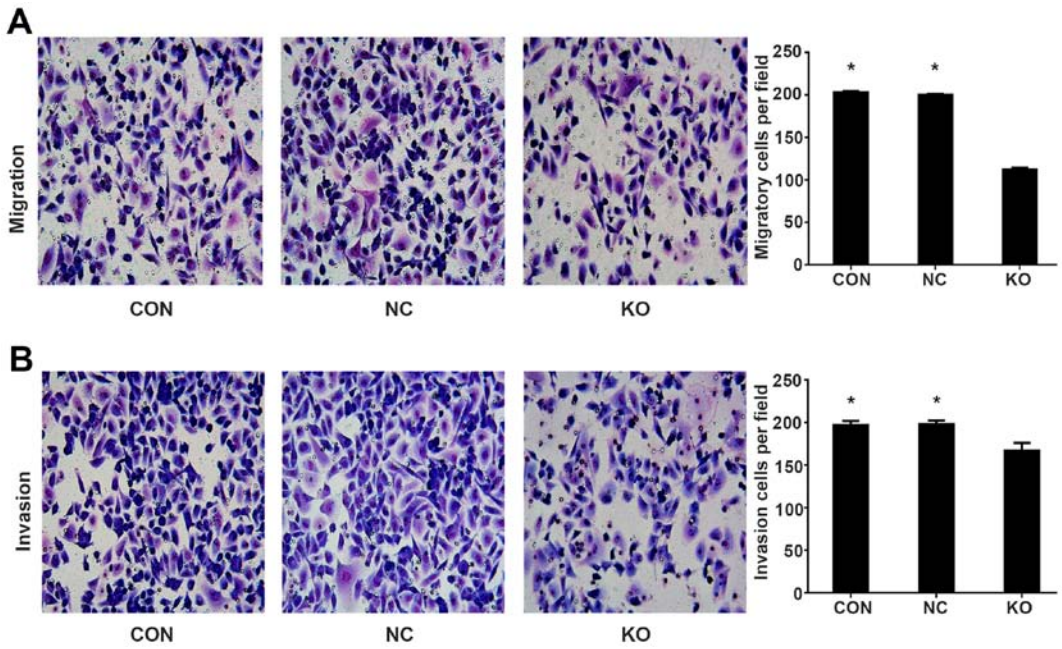


Figure 7. Effect of Rab11-FIP4 knockout on PANC-1 cell migration and invasion. (A) Transwell assay showed that Rab11-FIP4 knockout significantly depressed the migration of PANC-1 cells (P<0.05). (B) Transwell invasion assay indicated that Rab11-FIP4 knockout significantly inhibited the invasion of PANC-1 cells (P<0.05). The results are shown as the means ± SD. Representative photomicrographs of the Transwell results were obtained under x200 magnification. CON, no infection group. NC, negative control group. KO, Rab11-FIP4 knockout group. *P-value, compared with the KO, P<0.05.

that the effects of Rab11-FIP4 on cell growth contribute to alterations in cell proliferation, rather than the alterations in apoptosis. Cell cycle analysis further manifested a prominent decrease in stage S and G2/M in Rab11-FIP4 knockout cells, which showed that Rab11-FIP4 knockout induces cell cycle arrest in PANC-1 cells. Thus, Rab11-FIP4 knockout can suppress PANC-1 cell growth.

To further evaluate the impact of Rab11-FIP4 on PANC-1 cell invasion and metastasis, a Transwell assay was performed. The results indicated that knockout of Rab11-FIP4 suppressed the migratory and invasive ability of PANC-1 cells. Therefore, knockout of Rab11-FIP4 suppresses PANC-1 cell growth, invasion and metastasis. The underlying mechanisms need to be explored in further studies. The overexpression of Rab11-FIP4 may enhance PANC-1 cell growth, invasion and metastasis, leading to unfavourable outcomes in PC.

In conclusion, our findings suggest that Rab11-FIP4 exhibits tumor promotion and leads to a poor clinical outcome in PC. Rab11-FIP4 may be a novel target gene for pancreatic cancer therapy.

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