



ROCK1 regulates glycolysis in pancreatic cancer via the c-MYC/PFKFB3 pathway

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ABSTRACT

Background: Dysregulation of Rho-associated coiled coil-containing protein kinases (ROCKs) is involved in the metastasis and progression of various malignant tumors. However, how one of the isomers, ROCK1, regulates glycolysis in tumor cells is incompletely understood. Here, we attempted to elucidate how ROCK1 influences pancreatic cancer (PC) progression by regulating glycolytic activity.

Methods: The biological function of ROCK1 was analyzed *in vitro* by establishing a silenced cell model. Coimmunoprecipitation confirmed the direct binding between ROCK1 and c-MYC, and a luciferase reporter assay revealed the binding of c-MYC to the promoter of the PFKFB3 gene. These results were verified in animal experiments.

Results: ROCK1 was highly expressed in PC tissues and enriched in the cytoplasm, and its high expression was associated with a poor prognosis. Silencing ROCK1 inhibited the proliferation and migration of PC cells and promoted their apoptosis. Mechanistically, ROCK1 directly interacted with c-MYC, promoted its phosphorylation (Ser 62) and suppressed its degradation, thereby increasing the transcription of the key glycolysis regulatory factor PFKFB3, enhancing glycolytic activity and promoting PC growth. Silencing ROCK1 increased gemcitabine (GEM) sensitivity *in vivo* and *in vitro*.

Conclusions: ROCK1 promotes glycolytic activity in PC cells and promotes PC tumor growth through the c-MYC/PFKFB3 signaling pathway. ROCK1 knockdown can inhibit PC tumor growth *in vivo* and increase the GEM sensitivity of PC tumors, providing a crucial clinical therapeutic strategy for PC.

1. Introduction

Pancreatic cancer (PC) is a fatal disease, with a gradually increasing annual mortality rate approaching the annual incidence rate [1]. Statistics for 2020 show that the incidence of PC continues to increase, mainly in Western countries, and PC is expected to become the second leading cause of death among cancers by 2030 [2]. In the early stage of PC, often symptoms are not obvious, and it is difficult to detect. Patients tend to miss the optimal treatment period and thus lose the opportunity for radical surgery. The pancreas is hidden in the abdominal cavity and is close to major blood vessels, and accurate serum markers and imaging

models for early diagnosis are lacking, making the early detection of and screening of PC extremely difficult [3]. Additionally, PC shows significant resistance to radiotherapy and chemotherapy, resulting in a further increase in patient mortality. Surgical resection is still an effective treatment, but 80%–90% of patients have unresectable tumors, and even after successful resection, the 5-year survival rate of patients is only 10%–25% [4]. Therefore, understanding how the upstream and downstream molecular mechanisms of PC affect its occurrence and development may provide theoretical guidance for the clinical treatment of this deadly disease.

The mechanisms by which Rho-associated coiled coil-containing

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protein kinases (ROCKs) participate in tumor progression, especially in the stages of tumorigenesis, tumor development and metastasis, remain a focus of current research [5–7]. The ROCK isomer ROCK1 is highly expressed during breast cancer metastasis, thus enhancing the aggressiveness of cancer cells, and its overexpression was found to be significantly negatively correlated with patients survival [8,9]. High expression of ROCKs and high kinase activity have also been detected in patients with advanced breast cancer. A trend toward increased ROCK expression has also been observed in PC patients [10–12]. In a study of colorectal cancer, polymorphisms in ROCKs were found to be closely related to the progression of cancer [13,14]. High expression of ROCKs has also been detected in hepatocellular carcinoma [15]. However, the mechanism by which ROCK1 plays a role in regulating tumor glucose metabolism remains unclear.

MYC family oncoproteins, particularly c-MYC, are indispensable master regulators of metabolic reprogramming in various cancer types, including pancreatic cancer [16]. Studies have shown that c-MYC can participate in the regulation of tumor cell growth, cell cycle progression, metabolism, angiogenesis and other processes. However, the action of c-MYC alone does not seem to be the decisive factor in cancer. Instead, c-MYC must exert synergistic or antagonistic effects on other oncogenes and tumor suppressor genes to produce a series of effects [17]. The phosphorylation of c-MYC at certain sites governs its activation and subsequent biological functions through the transcriptional activation of target genes that are necessary for cell growth, and its phosphorylation at Ser 62 is essential for its oncogenic activity [18]. Previous studies have revealed that ROCK1 plays a crucial role in regulating prostate tumor growth through an interaction with c-MYC [19,20]. Therefore, the complex role of c-MYC in pancreatic cancer needs further study.

Glycolysis is a major metabolic pathway that provides energy requirements for tumor growth, leading to a high rate of glycolytic flux and a greater dependence on glucose in tumor cells [21]. The committed step in glycolysis is controlled by the enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), which converts fructose 6-phosphate (F6P) to fructose 2,6-diphosphate (F2,6BP). PFKFB3 is a major regulator of glycolysis in rapidly proliferating cells. Its high expression and/or dysregulation in multiple types of cancer has made PFKFB3 a potential therapeutic target [22].

In this paper, we showed that ROCK1 expression was significantly increased in PC tissues and that silencing ROCK1 can effectively inhibited the proliferation and migration of PC cells, promoted their apoptosis and increased their sensitivity to gemcitabine (GEM). ROCK1 was also confirmed to promote tumor growth through and increase sensitivity to GEM *in vivo* transplantation experiments. Mechanistically, ROCK1 could enhance the stability of c-MYC by increasing its phosphorylation at Ser 62 and inhibiting its degradation, further increasing the transcription and expression of the key glycolytic enzyme PFKFB3 thus promoting glycolytic activity in PC cells. These findings provide vital strategies for the treatment of PC mediated by ROCK1.

2. Materials and methods

2.1. Cell line maintenance and transfection

Human PC cell lines (AsPC-1, PANC-1, MIAPaCa-2, Capan-1, BxPC-3, and SW1990) and the normal pancreatic duct epithelial cell line HPDE6-C7 were purchased from The American Type Culture Collection (ATCC, VA, USA). These cell lines were cultured in Dulbecco's modified Eagle's medium with high glucose (Gibco Life Technologies, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco Life Technologies, NY, USA), 100 U/mL penicillin, and 100 g/mL streptomycin.

The si-ROCK1/NC plasmid and shROCK1/NC lentiviral plasmid were synthesized by Shanghai GenePharma (Shanghai, China). PC cells with a good growth status were seeded in 24-well plates or 6-well plates, transfected, and grown to approximately 70% confluence. Lipofectamine 2000 (Thermo Fisher Scientific, MA, USA) was used for

transfection. Transfection efficiency was verified by qRT-PCR at 24 h and by Western blotting at 48 h.

2.2. Clinical samples

Adjacent normal mucosa and tumor tissues were collected from PC patients after obtaining informed written consent and with the approval of the local medical ethics committees of Ruijin Hospital Affiliated with Shanghai Jiao Tong University.

2.3. RNA isolation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the treated group and the control group with TRIzol reagent, and the extracted RNA was then reverse transcribed into cDNA with a ChamQ SYBR qPCR Master Mix Kit (Vazyme Biotech, Jiangsu, China) and analyzed on a StepOne™ Real-Time PCR system (Thermo Fisher Scientific, MA, USA). The relative mRNA expression levels were normalized to those of GAPDH and were calculated using the comparative Cq method ($2^{-\Delta\Delta Cq}$) [20]. The primers used for RT-PCR were as follows: 5'-AACATGCTGCTGGATAAACTGG-3' (forward) and 5'-TGTATCACATCGTACCATGCCT-3' (reverse) for ROCK1; 5'-TTCCGTGTCCCCACTGCCAACGT-3' (forward) and 5'-CAAAGGTGGAGGAGTGGGTGTCGC' (reverse) for GAPDH; 5'-GCCCGTGAGGCAGAGGCTGC-3' (forward) and 5'-TGGTGAGGACGATTATGGCCC-3' (reverse) for PFKM2; 5'-GCCATCCTGCAACACTTAGGGC TTGAG-3' (forward) and 5'-GTGAGGATGTAGCTTGATAGGGTCCC-3' (reverse) for HK2; 5'-ATGGCAACTCTAAGGATCAGC-3' (forward) and 5'-CCAACCCAAACAACGTATCT-3' (reverse) for LDHA; 5'-TCCATGT-GACCATGAGGAATG-3' (forward) and 5'-TCGGCTAGTTAGGGTACACTTC-3' (reverse) for HIF-1 α ; 5'-ATTGCGGTTTCGATGCCAC-3' (forward) and 5'-GCCACAAACTGTAGGGTCTG-3' (reverse) for PFKFB3; and 5'-AAACACAAACTTGAACAGCTAC-3' (forward) and 5'-ATTT-GAGGCAGTTACATTATGC-3' (reverse) for c-MYC.

2.4. Immunofluorescence assay (IF) assay

Cell suspensions were inoculated in confocal dishes. When the cells were 60%–70% confluent, the medium was discarded, and the cells were fixed with 4% paraformaldehyde for 30 min. Then, the cells were washed with PBS, permeabilized with 0.2% Triton X-100 (Shanghai Genbase Gene-Tech, Shanghai, China) for 30 min, washed with PBS, and further blocked with a 5% BSA solution at room temperature for 30 min. The cells were incubated with the primary antibody at 4 °C overnight. After rewarming, the cells were washed with PBS, a fluorescent secondary antibody (ProteinTech Group, IL, USA) was added, and the cells were incubated for 3 h at room temperature in the dark. Nuclei were stained with DAPI staining solution (Beyotime Biotechnology, Shanghai, China) for 30 min. The cells were washed with PBS, observed under a laser confocal microscope (Olympus Corporation, Tokyo, Japan) and photographed [23].

2.5. MTT assay

Cells were seeded in a 96-well plate at a density of 5×10^4 cells/mL, with 100 μ L of cell suspension per well and 4 wells per group. After 6, 24, 48 and 72 h of culture, the cell density was examined under a light microscope (Nikon Corporation, Tokyo, Japan), and the cells were photographed. Then, 10 μ L of MTT solution (Sigma-Aldrich, MO, USA) was added to each well in the dark, and the culture was continued for 4 h. After discarding the medium, 150 μ L of DMSO was added to each well, and the plate was incubated with gentle shaking for 10 min. Then, the absorbance of each group was measured with a microplate reader (Molecular Devices, CA, USA). The detection wavelength was 492 nm.

2.6. Colony formation assay

After transfection, AsPC-1 and PANC-1 cells were digested with trypsin, inoculated in a 6-well plate at a density of 600 cells/mL and incubated at 37 °C for 10 days. The original medium was discarded, and the cells were washed with PBS twice and fixed with 4% paraformaldehyde for 30 min. The fixative was discarded, and the cells were washed with PBS twice. One milliliter of 0.1% crystal violet staining solution (Beijing Solarbio Science & Technology, Beijing, China) was added to each well and the cells were stained for 20 min. The staining solution was discarded, and the cells were washed with PBS 3–5 times until the culture plate had no background color. The colonies that formed were counted and photographed.

2.7. Apoptosis assay

Cells were seeded into 6-well plates at a density of 3×10^5 cells/mL. Transfection was performed at approximately 70% confluence, and then the cells were cultured for 48 h. The cell culture medium was discarded, and the cells were digested with trypsin (without EDTA). The trypsin was discarded, and the cells were washed twice with PBS by centrifugation at 400 × g for 5 min. After the supernatant was discarded, 500 μL of binding buffer was added to each tube in the dark to resuspend the cells. Then, the cells were gently mixed with 5 μL of Annexin V-FITC, and finally, 5 μL of propidium iodide (Vazyme Biotech, Jiangsu, China) was added. The reaction was allowed to proceed in the dark for 10 min. The stained cells were captured using a flow cytometer (Becton, Dickinson and Company, NY, USA).

2.8. Transwell invasion assay

Cells were suspended in serum-free basal medium at a density of 2.5×10^5 cells/mL. Two hundred microliters of the suspension was added to the upper chamber (Corning Incorporated, NY, USA), and 500 μL of medium containing serum was added to the lower chamber and cultured for 24 h. The liquid in the chamber was discarded, and the cells were washed twice with PBS. The cells were fixed with 500 μL of 4% paraformaldehyde for 30 min in a 24-well plate, washed with PBS twice, and then air-dried appropriately. The cells were stained with 300 μL of a 0.1% crystal violet solution for 20 min and washed with PBS twice. Then, the membrane in the upper chamber was wiped with a cotton swab to remove the remaining cells, washed with PBS 3 times, and air dried at room temperature for 5 min. Cell migration was observed under a microscope with a 100× objective (Olympus, Tokyo, Japan) and photographed.

2.9. Glucose uptake and lactate release assays

Cell suspensions were seeded into 96-well plates at a density of 5×10^4 cells/mL, with 100 μL of cell suspension per well and 3 wells per group. The cell culture medium was removed for detection after 6, 24 and 48 h of culture. The glucose concentration in the medium was measured using a glucose determination kit (Shanghai Rongsheng BIOTECH, Shanghai, China), and the lactic acid concentration was measured according to the instructions of the lactic acid test kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) [24]. The tissue was homogenized at a ratio of 0.1 g of tumor tissue to 1 mL of prechilled lactate assay buffer on ice. The homogenate was centrifuged at 12,000 × g for 5 min at 4 °C, after which the supernatant was collected for analysis. The lactic acid concentration was measured using the lactic acid test kit according to the manufacturer's instructions.

2.10. ECAR assays

Metabolic indicators (ECAR) were used to evaluate glycolytic flux. The treated cells were evenly spread on a 96-well XF cell culture plate

(Agilent Technologies Inc., CA, USA) at a density of 5×10^4 cells/mL, with 80 μL of cell suspension per well and 4 wells per group. After 48 h of cultivation, the culture medium was discarded, and fresh assay medium containing different detection reagents was added to each well. The cartridge was loaded with glucose (10 mM), oligomycin (1 μM), and 2-deoxyglucose (2-DG, 50 mM) to detect the ECAR using a Seahorse XFe96 Cell Energy Metabolism Analyzer (Agilent Technologies Inc., CA, USA) at the specified time points.

2.11. Western blotting

Cells were lysed with RIPA buffer, and the protein concentration was measured with a BCA kit (Beyotime Biotechnology, Shanghai, China). The primary antibodies used were as follows: rabbit anti-ROCK1, rabbit anti-β-actin, rabbit anti-GAPDH, rabbit anti-c-MYC, rabbit anti-p-c-MYC (Ser 62), and rabbit anti-PFKFB3 (1:1000; all from ZEN-BioScience).

2.12. Coimmunoprecipitation (Co-IP) assay

Transfected AsPC-1 cells were cultured for 48 h, 200 μL of precooled NP40 lysate (Beyotime Biotechnology, Shanghai, China) was added to each well, and the cells were lysed for 30 min with vortexing every 10 min. The supernatant was centrifuged at 13800 × g for 10 min at 4 °C. Then, 150 μL of cell supernatant was removed as the input sample. Ten microliters of Protein A/G agarose beads (Selleck Chemicals, TX, USA) was added to the IgG sample, and the target protein sample was incubated with slow rotation at 4 °C for 2 h. The protein samples were recovered, and the magnetic beads were discarded. The corresponding antibody (2 μg) was added to each tube, and the tubes were incubated overnight at 4 °C with slow rotation. Magnetic beads (30 μL) were added to the IgG tube and the target protein tube, and the tubes were incubated at 4 °C with slow rotation for 2 h. The samples were washed 5 times on the magnetic rack with NP40 containing PMSF (Beyotime Biotechnology, Shanghai, China) for 2 min each time, and 50 μL of sample was reserved during the last cleaning step. Loading buffer (5×) was added to the samples which were subsequently boiled for 5 min. The IgG group and target protein group were prepared as described and stored at –80 °C for later use. The following antibodies were used: rabbit anti-ROCK1 (ZEN-BIOSCIENCE); Alexa Fluor 488 (Fcmacs Biotech, Jiangsu, China); DAPI, mouse anti-c-MYC (Beyotime Biotechnology, Shanghai, China); and rhodamine (TRITC)-conjugated goat anti-mouse IgG (H + L) (1:50).

2.13. Luciferase reporter assay

Cells were seeded in 24-well plates at a density of 5×10^4 cells/mL, and transfection began when the confluence reached approximately 70%. The cells were divided into three groups according to the type of transfected plasmid, with three replicates per group. The first group was transfected with the pcDNA3.1(+)-NC and pGL3-basic-PFKFB3 pro (–2000 to –1800) plasmids. The second group was transfected with pcDNA3.1(+)-c-MYC and pGL3-basic-PFKFB3 pro (–2000 to –1800). The third group was transfected with the plasmids pcDNA3.1(+)-c-MYC and pGL3-basic-mutPFKFB3 pro (–2000 to –1800) plasmids. At the same time, each group was transfected with a Renilla plasmid as an internal reference. The dose ratio of the three plasmids was 1:1:1, and the total amount of plasmids per well was 0.2 μg. The samples were collected after 48 h. Luciferase activity was measured using the instructions for the luciferase reporter assay kit (Vazyme Biotech, Jiangsu, China) with a full wavelength scanner (Thermo Fisher Scientific, MA, USA) according to the manufacturers' instructions [25].

2.14. Phosphofructokinase enzymatic activity assay

AsPC-1 and PANC-1 cells were seeded into 6-well plates at a density of 2×10^5 cells/mL. After adherence, the ROCK1 inhibitor GSK429286A

(MCE, 10 μ M) was added, and samples were collected at 12 h, 24 h and 48 h after treatment. According to the instructions of the phosphofructokinase test kit (Nanling Jiancheng Bioengineering Institute, Jiangsu, China), the cells were lysed by ultrasonication and centrifuged at 4 °C and 8000 $\times g$ for 10 min, after which the supernatant was collected for detection. After adding the specified amount of working solution, the samples were quickly analyzed using a spectrophotometer (Thermo Fisher Scientific, MA, USA), and the OD₃₄₀ value of each sample was recorded.

2.15. Xenograft assay

Male BALB/c nude mice were housed in a specific pathogen-free (SPF) laminar flow cabinet in the animal experimental center, with 5 mice per cage [26]. The feeding temperature was 22 ± 1 °C, and the animals were exposed to light for 12 h/day. The food and water were sterilized in advance, and the mice could ingest them freely. All experiments were conducted in accordance with the regulations of the Ethics Committee for Animal Experiments.

AsPC-1 cells transduced with shNC and shROCK1 lentiviral plasmids were digested with trypsin and washed with PBS three times by centrifugation at 200 $\times g$ for 5 min each. The cells were suspended in normal saline, and the density was adjusted to 6 × 10⁶ cells/mL. Each nude mouse was injected subcutaneously with 200 μ L of the cell suspension, with 7 mice per group. The nude mice were weighed every four days, and the length and width of the tumors were accurately measured with Vernier calipers when obvious axillary tumors appeared. The tumor volume was calculated using the formula (length × width²) / 2. Beginning on the 20th day after cell inoculation, the nude mice were intraperitoneally injected with GEM (MedChemExpress, NJ, USA) (25 mg/kg, 50 mg/kg) every Monday and Thursday. On the 44th day, all mice in the experiment were euthanized by cervical dislocation. The tumors were dissected, weighed and photographed, and the tumor inhibition rate was calculated. Some tumor tissues were soaked in 4% paraformaldehyde for immunohistochemical analysis. The following antibodies were used: rabbit anti-ROCK1, rabbit anti-p-c-MYC (Ser 62) (Zen-Bioswisch), and rabbit anti-PFKFB3 (Beijing Solarbio Science & Technology, Beijing, China) (1:50).

3. Results

3.1. High ROCK1 expression is associated with shorter survival of pancreatic cancer patients

The expression level of ROCK1 in PC was investigated with the GEPIA database (<http://gepia.cancer-pku.cn/>). The ROCK1 mRNA level was abnormally high in the tissues of PC patients ($p < 0.05$, Fig. 1A). The ROCK1 protein level in PC tissues and adjacent tissues was further analyzed by performing immunohistochemical staining of clinical samples, and the results were consistent with those of the database analysis (Fig. 1B). Then, the GEPIA database was utilized to predict the relationship between the ROCK1 expression level and overall survival rate of patients with PC. The ROCK1 expression level was found to be closely related to patient prognosis of patients. As the ROCK1 expression level increased, the overall survival rate of patients with PC decreased significantly ($p = 0.025$, Fig. 1C), indicating a poor prognosis. Then, the expression of ROCK1 in six PC cell lines was analyzed using qRT-PCR. Compared with those in HPDE6-C7 normal pancreatic duct epithelial cells, ROCK1 mRNA and protein levels were significantly higher in AsPC-1 and PANC-1 cells and lower in Capan-1, MIAPaCa-2, BxPC3 and SW1990 cells (Fig. 1D, E). The above results indicated that the expression of ROCK1 differed among PC cell lines. Although more PC cells in the screen showed a trend toward low ROCK1 expression, AsPC-1 and PANC-1 cells were derived from a primary pancreatic head tumor and an *in situ* pancreatic head tumor, respectively. These cells have greater metastatic ability. Therefore, they still have research value and

significance. Since the subcellular localization of the ROCK1 protein is closely related to its function, the specific localization of ROCK1 in PC cells was explored. Due to the relatively high expression levels of ROCK1 in AsPC-1 and PANC-1 cells, these two cell lines were selected for immunofluorescence staining. ROCK1 was concentrated in the cytoplasm of AsPC-1 and PANC-1 cells, and only a small amount was distributed in the nucleus (Fig. 1F). These above results suggest that abnormally high expression of ROCK1 is negatively correlated with the overall survival rate of patients with PC. Thus, ROCK1 has obvious prognostic value and could be considered an oncogenic factor for further study.

3.2. Silencing ROCK1 inhibits the proliferation and migration of pancreatic cancer cells and promotes apoptosis

As a method to confirm the hypothesis that ROCK1 plays a role as an oncogenic factor in the progression of PC, two cell lines with high expression of ROCK1, AsPC-1 and PANC-1, were selected to construct a ROCK1 silencing model. Three siRNA-ROCK1 sequences were designed and their silencing efficiencies were compared. The ROCK1-Homo-1410 plasmid (si-ROCK1-2) was found to have the best ROCK1 silencing efficiency in the two PC cell lines. The qRT-PCR results showed that the silencing efficiency in AsPC-1 and PANC-1 cells was 89.1% ($p < 0.001$, Fig. 2A) and 61.8% ($p < 0.001$, Fig. 2B), respectively. Western blotting was subsequently employed to further confirm the silencing effect. The ROCK1-Homo-1410 plasmid had the best silencing efficiency (Fig. 2C, D); thus, this plasmid was selected to construct the ROCK1 silencing model for subsequent experiments.

Then, the effect of ROCK1 silencing on the biological behavior of PC cells, including cell proliferation, apoptosis and migration, was evaluated. First, the effect of ROCK1 on the proliferation of PC cells was analyzed. After transfection with the ROCK1-Homo-1410 plasmid, the cells were cultured for 6, 24, 48 and 72 h, and proliferation was evaluated using an MTT assay. The growth of AsPC-1 and PANC-1 cells was significantly inhibited after culture for 72 h, with inhibition rates of up to 29.8% and 9.7%, respectively (Fig. 2E, F), suggesting that ROCK1 silencing can effectively suppress the proliferation of these two PC cell lines, with a more obvious effect on AsPC-1 cells.

The impact of ROCK1 on PC cell colony formation was evaluated by performing a colony formation assay. As shown in Fig. 2G and H, after silencing ROCK1, colony formation by AsPC-1 and PANC-1 cells was significantly inhibited, with inhibition rates of 51.3% and 15.8%, respectively. The function of ROCK1 in PC cell apoptosis was further explored using flow cytometry. ROCK1 silencing promoted the apoptosis of AsPC-1 and PANC-1 cells, with a more obvious effect on AsPC-1 cells. Compared with that in the control group, after ROCK1 silencing, the total percentage of apoptotic AsPC-1 cells (1.1% vs. 25.0%) (Fig. 2I) and PANC-1 cells (8.0% vs. 10.7%) increased (Fig. 2J). A Transwell assay was employed to investigate the effect of ROCK1 on the migration of the two PC cell lines. After ROCK1 was silenced, the cells were cultured for 24 h in a Transwell chamber to evaluate cell migration. The migration of AsPC-1 and PANC-1 cells was significantly suppressed by ROCK1 silencing, with inhibition rates of 61.6% and 37.9%, respectively (Fig. 2K). These results suggest that ROCK1 plays a positive regulatory role in the occurrence and development of PC.

3.3. ROCK1 enhances glycolytic activity in pancreatic cancer cells

Tumor cells utilize glycolysis as their main energy source even under sufficient oxygen, a phenomenon called the “Warburg effect”. The cytoplasm is the main site of glycolysis in tumor cells. Therefore, we hypothesized that ROCK1 enrichment in the cytoplasm is related to glycolysis in tumor cells and that it plays a role as an oncogenic factor by promoting glycolytic activity. The effects of ROCK1 on glucose uptake and lactate release in PC cells were examined. The glucose intake and lactic acid release of AsPC-1 and PANC-1 cells were significantly

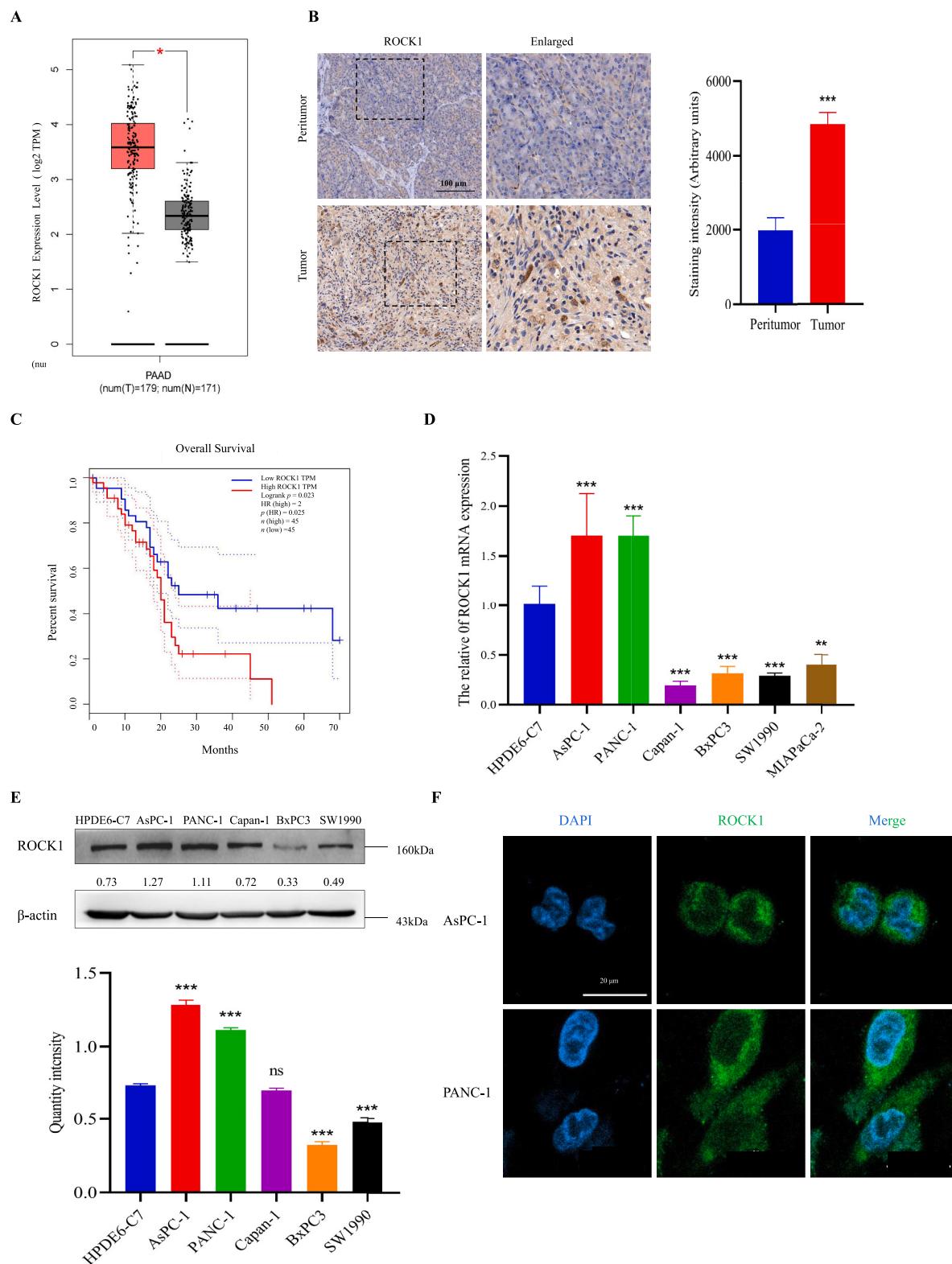
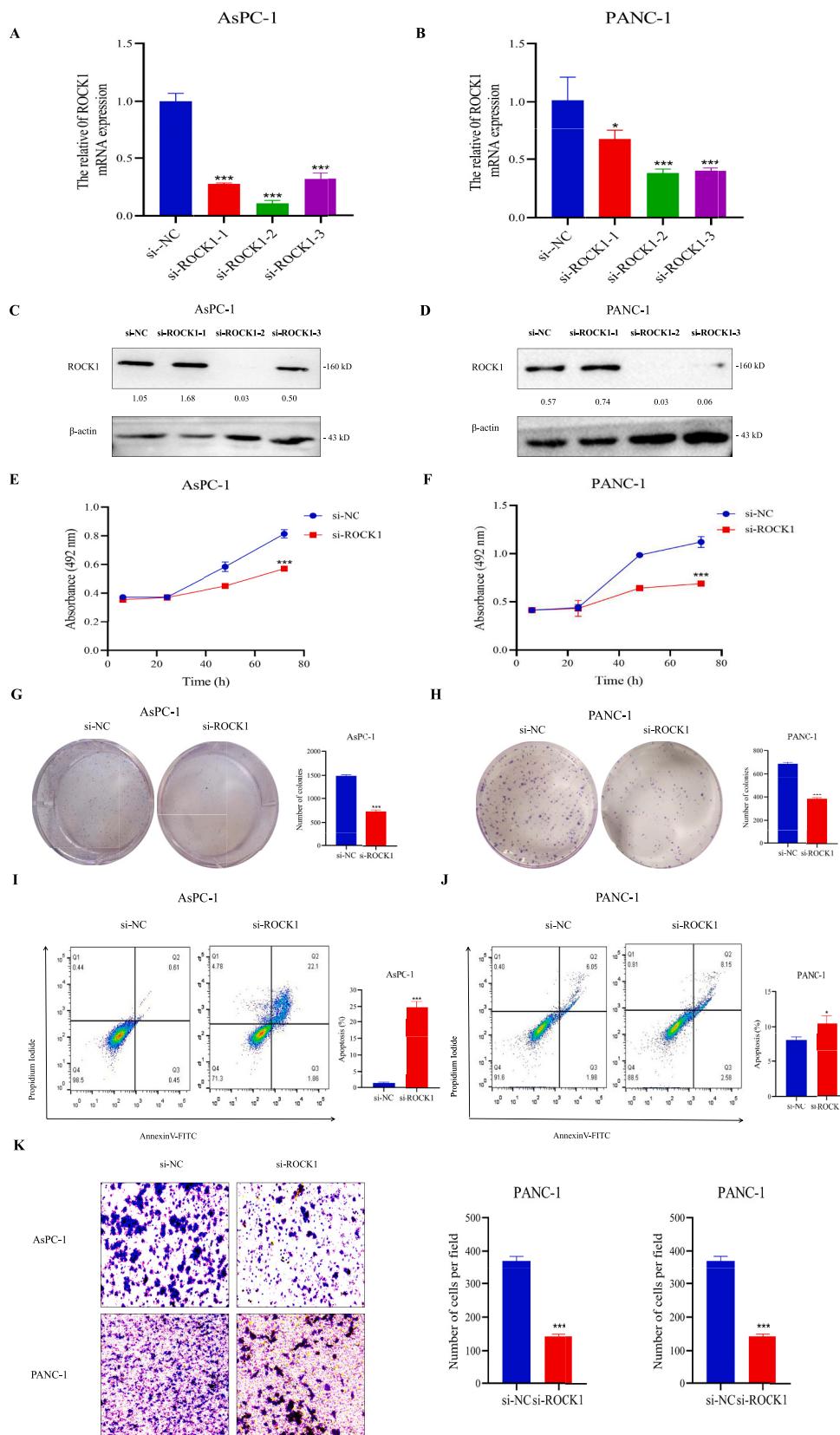


Fig. 1. High ROCK1 expression is associated with shorter survival of pancreatic cancer patients. **A** ROCK1 mRNA expression in pancreatic cancer tissues and paracancerous tissues predicted by the GEPIA database, with red representing cancer tissues and black representing paracancerous tissues. (* $p < 0.05$). **B** Immunohistochemical analysis of ROCK1 protein expression in pancreatic cancer clinical samples ($n = 5$, left panel: $40\times$, right panel: $100\times$, *** $p < 0.001$). **C** Relationship between the ROCK1 expression level and overall survival rate of patients analyzed using the GEPIA database. (* $p < 0.05$) **D** qRT-PCR detection of the relative expression of ROCK1 in six pancreatic cancer cell lines (HPDE6-C7 group as a reference, $n = 4$, ** $p < 0.01$, *** $p < 0.001$). **E** WB analysis of the relative expression of ROCK1 in five pancreatic cancer cell lines (HPDE6-C7 group as a reference, $n = 4$, *** $p < 0.001$). **F** ROCK1 localization in AsPC-1 and PANC-1 cells was detected using an immunofluorescence assay ($n = 3$, $400\times$). (** $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



(caption on next page)

Fig. 2. Silencing ROCK1 inhibits the proliferation and migration of pancreatic cancer cells and promotes apoptosis. A, B The silencing efficiency of ROCK1 in AsPC-1 and PANC-1 cells was measured using qRT-PCR ($n = 3$, * $p < 0.05$, *** $p < 0.001$). C, D Western blot showing the effect of ROCK1 silencing on in AsPC-1 and PANC-1 cells ($n = 3$). E, F MTT assay to determine the effect of ROCK1 silencing on the proliferation of AsPC-1 and PANC-1 cells ($n = 4$, *** $p < 0.001$). G, H Colony formation assays showing the effect of ROCK1 silencing on the ability of AsPC-1 and PANC-1 cells to form colonies ($n = 4$, *** $p < 0.001$). I, J The apoptotic rate of AsPC-1 and PANC-1 cells was measured using an Annexin V (AV) and propidium iodide (P) apoptosis detection kit. The percentages of apoptotic cells are shown. The data in the flow cytometry plots are representative images, while the data in the bar charts represent the overall data. Thus, the data in the plots and graphs do not necessarily represent each other ($n = 3$, * $p < 0.05$, *** $p < 0.001$). K The migration of AsPC-1 and PANC-1 cells was assessed using a Transwell assay (** $p < 0.001$).

reduced after ROCK1 silencing for 48 h. Compared with those of the control group, the glucose uptake rate of AsPC-1 cells decreased (21.3% vs. 13.4%) after silencing ROCK1 (Fig. 3A). The glucose uptake rate of PANC-1 cells was also decreased compared to that in the control group (12.7% vs. 9.7%) (Fig. 3B). After silencing ROCK1, the lactate release rates of AsPC-1 and PANC-1 cells were decreased by 31.0% and 25.8%, respectively (Fig. 3C, D). Compared with control cells, ROCK1-silenced cells exhibited reduced levels of ECAR. Consequently, ROCK1 deficiency inhibited glycolysis while reducing the glycolytic capacity and the glycolytic reserve in AsPC-1 and PANC-1 cells, and these processes were decreased in AsPC-1 and PANC-1 cells by 50.0% and 55.5%, 47.5% and 43.6%, 45.9% and 42.0%, respectively (Fig. 3E, F). These results indicated that ROCK1 can promote glycolytic activity in PC cells.

Changes in the expression of related genes, including PKM2, HK2, LDHA, PFKFB3 and the related transcription factors HIF-1 α and c-MYC, were measured using qRT-PCR after ROCK1 silencing to identify the mechanism by which ROCK1 promotes glycolysis in PC cells. As shown in Fig. 3G, H, HK2, LDHA, PFKFB3 and c-MYC were downregulated in both AsPC-1 and PANC-1 PC cells after ROCK1 silencing. Among these genes, the expression of the PFKFB3 ($p < 0.001$, $p < 0.05$) and c-MYC ($p < 0.05$, $p < 0.01$) mRNAs was significantly downregulated in both PC cell lines. Further analysis using the GEPIA database revealed that ROCK1 gene expression was significantly positively correlated with the expression of the PFKFB3 and c-MYC mRNAs (Fig. 3I, J). The aforementioned data suggest that ROCK1 can promote glycolytic activity in PC cells by increasing the mRNA expression levels of PFKFB3, a key enzyme promoting glycolysis, and c-MYC, a transcription factor associated with glycolysis.

3.4. ROCK1 directly binds to c-MYC and promotes its phosphorylation (Ser 62) but does not directly affect the kinase activity of PFKFB3

After the correlations between ROCK1 mRNA levels and c-MYC and PFKFB3 mRNA levels were confirmed, the influence of ROCK1 silencing on PFKFB3 and c-MYC protein levels was further evaluated. Western blotting indicated that the total c-MYC protein level was decreased in both AsPC-1 and PANC-1 cells after silencing ROCK1. An analysis of phosphorylated c-MYC (Ser 62) revealed that after ROCK1 silencing, the levels of phosphorylated c-MYC (Ser 62) and PFKFB3 proteins were significantly decreased in both PC cell lines (Fig. 4A, B). Since ROCK1 itself acts as a kinase, we speculated that its enzymatic activity may also affect the expression of downstream proteins. In this study, the two PC cell lines were treated with the ROCK1 inhibitor GSK429286A. At a GSK429286A concentration of 20 μ M, the protein levels of phosphorylated c-MYC (Ser 62) and PFKFB3 were effectively decreased in both PC cell lines (Fig. 4C, D). These results showed that both the high protein expression and increased enzymatic activity of ROCK1 promoted the protein expression of the key glycolytic enzyme PFKFB3 and the phosphorylation of the transcription factor c-MYC (Ser 62) in PC cells, thus increasing glycolytic activity in PC cells.

We speculated that the increase in the kinase activity of ROCK1 may enhance the enzymatic activity of the downstream enzyme PFKFB3, thus promoting tumor glycolysis. Then, the two PC cell lines were treated with 10 μ M GSK429286A to evaluate the kinase activity of PFKFB3. No significant difference in intracellular PFKFB3 kinase activity was observed between AsPC-1 cells and control cells after 12 h of treatment. Although intracellular PFKFB3 kinase activity decreased after 24 h of treatment, it was slightly increased after 48 h of treatment (Fig. 4E).

Similarly, the intracellular kinase activity of PFKFB3 increased in PANC-1 cells after 12 h and 48 h of treatment with the ROCK1 inhibitor (Fig. 4F). These results suggested that the decrease in ROCK1 kinase activity itself did not directly lead to a the decrease in downstream PFKFB3 kinase activity. Since ROCK1 had no direct effect on the kinase activity of PFKFB3, we aimed to determine whether they directly interacted at the protein level. If not, does the transcription factor c-MYC act as the bridge between these proteins? The interaction between ROCK1 and PFKFB3 was predicted using the protein interaction database STRING (<https://cn.string-db.org/>). The results indicated no direct binding between the ROCK1 and PFKFB3 proteins. However, direct binding interactions might occur between the ROCK1 and c-MYC proteins and between the c-MYC and PFKFB3 proteins (Fig. 4G). A co-IP assay was applied to verify these predictions in AsPC-1 cells. No direct binding between the ROCK1 and PFKFB3 proteins was detected, but a direct and strong binding interaction between the ROCK1 and c-MYC proteins was detected. In addition, the results of the IF assay showed that c-MYC colocalized with ROCK1 in the cytoplasm.

3.5. ROCK1 promotes glycolytic activity in pancreatic cancer cells through the c-MYC/PFKFB3 signaling axis

Phosphorylation of c-MYC (Ser 62) inhibits its degradation via the ubiquitin-proteasome pathway, thereby increasing the stability of the c-MYC protein. Thus, we speculated that ROCK1 could enhance the stability of c-MYC, thus promoting the transcription of downstream target genes. AsPC-1 cells were transfected with the ROCK1 silencing plasmid, cultured for 48 h, and then treated with 10 μ M MG-132 (a proteasome inhibitor) for 4 h. Compared with the untreated siNC group, MG132 treatment significantly increased the accumulation of the c-MYC protein, indicating that c-MYC was indeed degraded through the proteasome pathway. However, when ROCK1 was silenced, the accumulation of the c-MYC protein in the MG132-treated group was still lower than that in the untreated siNC group (Fig. 5A). Furthermore, CHX (10 mg/mL, a protein synthesis inhibitor) was used to treat ROCK1-silenced AsPC-1 cells, and samples were collected for Western blot analysis after 2 h, 4 h and 8 h of treatment. The experimental results suggested that the c-MYC protein began to degrade significantly at the 4th hour in the control group but was obviously degraded at the 2nd hour after ROCK1 silencing, further indicating the stabilizing and protective effect of ROCK1 on the c-MYC protein (Fig. 5B). These results suggest that ROCK1 binds to the c-MYC protein in the cytoplasm to promote the phosphorylation of c-MYC (Ser 62), thereby enhancing its stability and inhibiting its degradation via the proteasome pathway.

The relationship between c-MYC and the downstream enzyme PFKFB3 was further explored. An analysis of the GEPIA database revealed a significant positive correlation between the expression of these two genes (Fig. 5C). Because c-MYC can promote the transcription of downstream genes, the possible binding site of c-MYC on the promoter region of the PFKFB3 gene was further predicted using the JASPAR and NCBI databases (relative score = 0.85568) as GGGCATGTGCTC (Fig. 5D). This relationship was verified by conducting a luciferase reporter assay. After transfection of the corresponding plasmids into HEK293T cells, the luciferase activity of the groups transfected with the pGL3-basic-PFKFB3 pro (-2000 to -1800) and pcDNA3.1(+)-c-MYC plasmids was significantly increased (1.5-fold) compared with that in the control group transfected with the pGL3-basic-PFKFB3 pro (-2000 to -1800) and pcDNA3.1(+)-NC plasmids. In cells transfected with

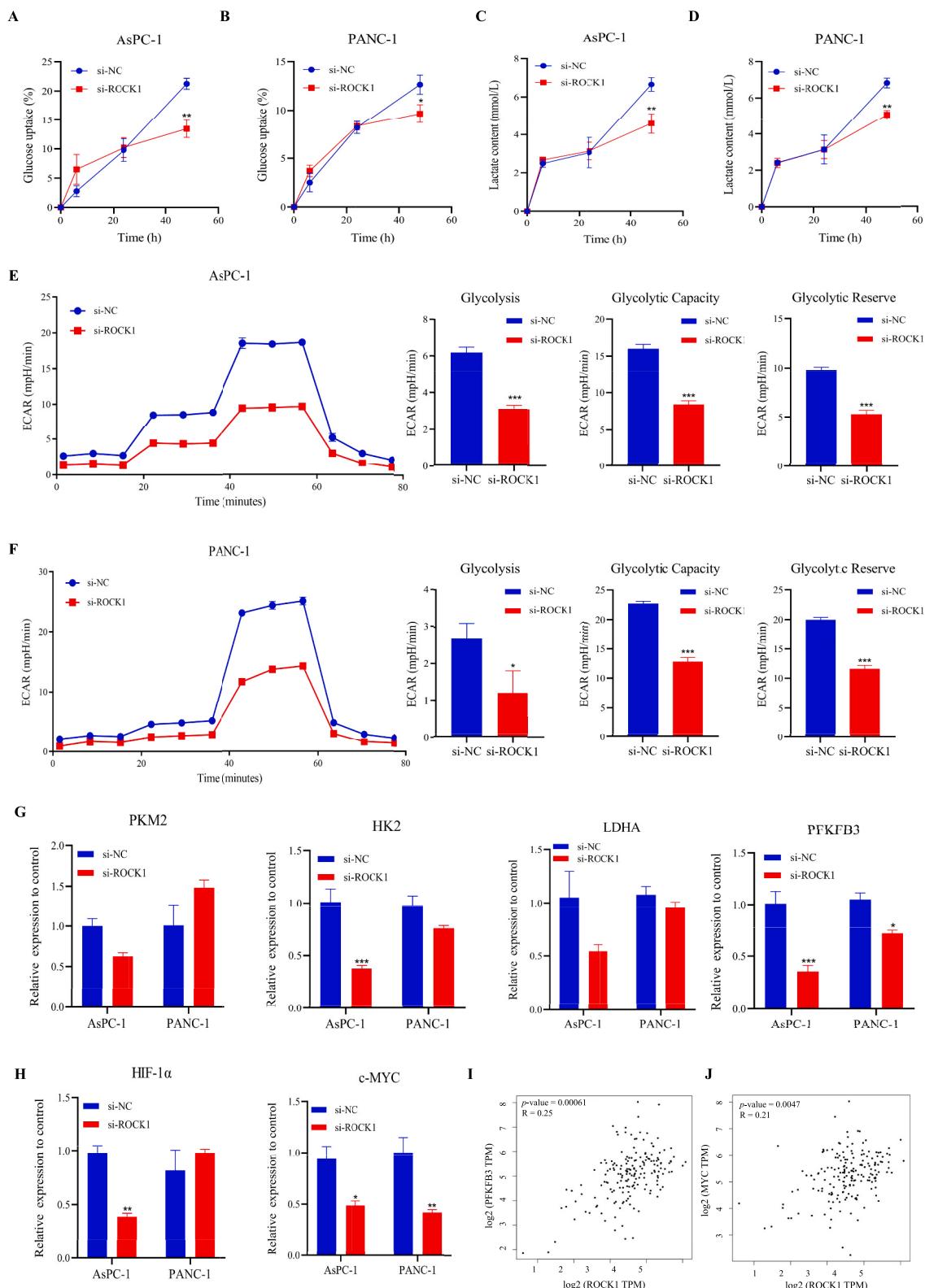


Fig. 3. *ROCK1* enhances glycolytic activity in pancreatic cancer cells. **A, B** Quantitative map of the glucose uptake rate in AsPC-1 and PANC-1 cells ($n = 3$, $*p < 0.05$, $**p < 0.01$). **C, D** Quantification of lactic acid release from AsPC-1 and PANC-1 cells ($n = 3$, $**p < 0.01$). **E, F** *ROCK1*-knockdown AsPC-1 and PANC-1 cells were transferred to Seahorse XF24 cell culture microplates for ECAR assays. Quantification of glycolysis, the glycolytic capacity and glycolytic reserve in the ECAR assays ($n = 4$, $*p < 0.05$, $***p < 0.001$). **G, H** qRT-PCR was used to detect the expression of *ROCK1*-related glycolysis genes in pancreatic cancer cells ($n = 3$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). **I, J** The correlations between *ROCK1* and *PFKFB3* and between *ROCK1* and *c-MYC* were analyzed using the GEPIA database. ($**p < 0.01$, $***p < 0.001$).

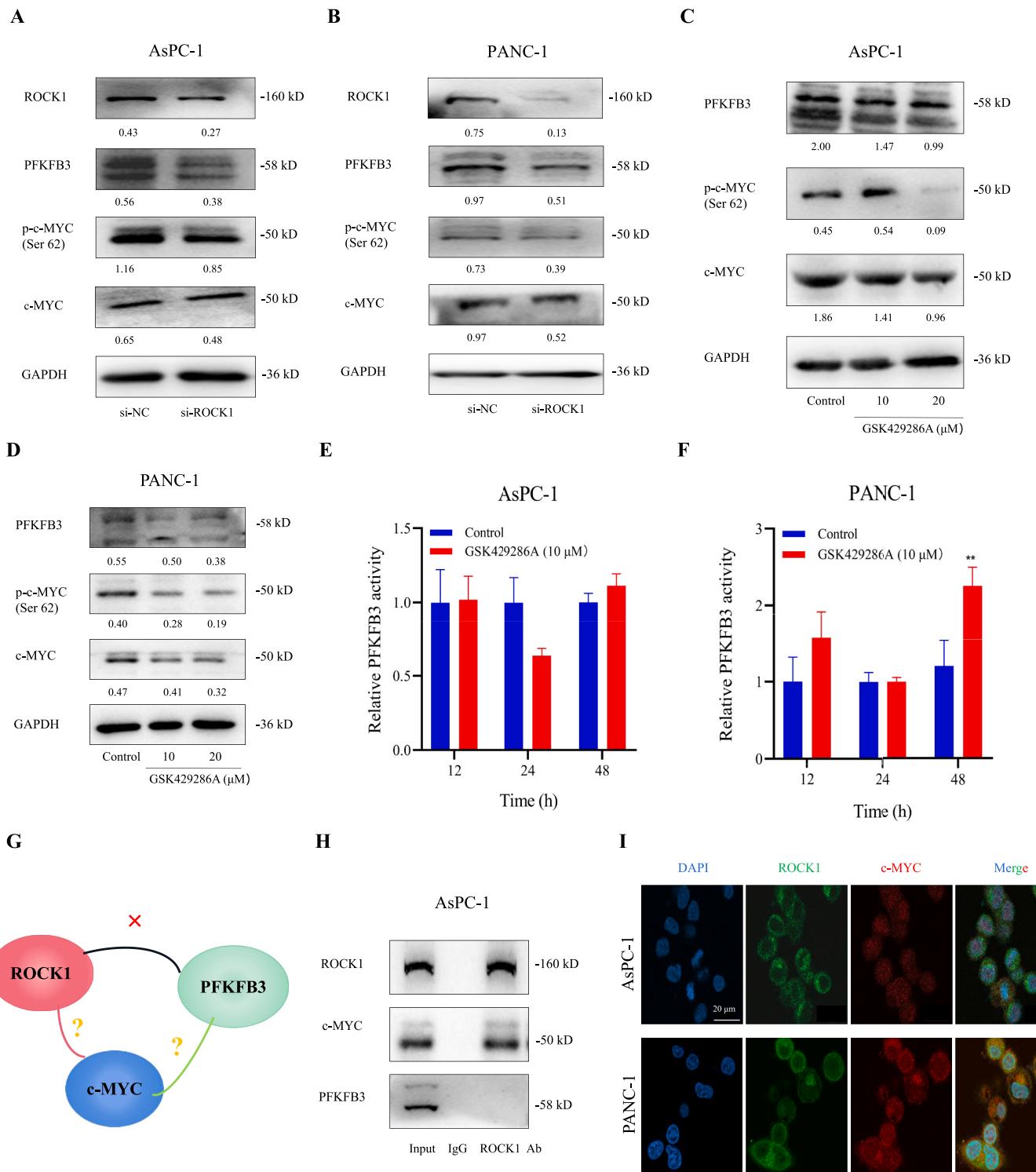


Fig. 4. ROCK1 directly binds to c-MYC and promotes its phosphorylation (Ser 62) but does not directly affect the kinase activity of PFKFB3. **A-D** Western blotting was used to detect the effects of ROCK1 silencing or ROCK1 kinase inhibitor treatment on glycolysis-related proteins in AsPC-1 and PANC-1 cells ($n = 3$). **E, F** The effects of ROCK1 kinase activity on PFKFB3 kinase activity were detected by performing phosphofructokinase enzyme activity assays ($n = 3$). **G** The relationships among ROCK1, c-MYC and PFKFB3 proteins were analyzed via the STRING database. **H** The direct binding of the ROCK1 protein to the c-MYC and PFKFB3 proteins was verified by co-IP ($n = 3$). **I** ROCK1 and c-MYC localization in cells was detected using immunofluorescence staining ($n = 3$, $400 \times$) ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

pGL3-basic-mutPFKFB3 pro (-2000 to -1800) and pcDNA3.1(+)-c-MYC, the luciferase activity decreased significantly compared with that in the experimental group. These results indicated that c-MYC bound to the promoter of the PFKFB3 gene and promoted its transcription. Further validation was performed in two PC cell lines, AsPC-1 and

PANC-1. With an increase in the quantity of pcDNA3.1(+)-c-MYC plasmid transfected, a dose-dependent increase in luciferase activity was observed (Fig. 5E). These results indicated that c-MYC can promote the transcription of the PFKFB3 gene in a dose-dependent manner, thus increasing the expression of the PFKFB3 protein. AsPC-1 and PANC-1

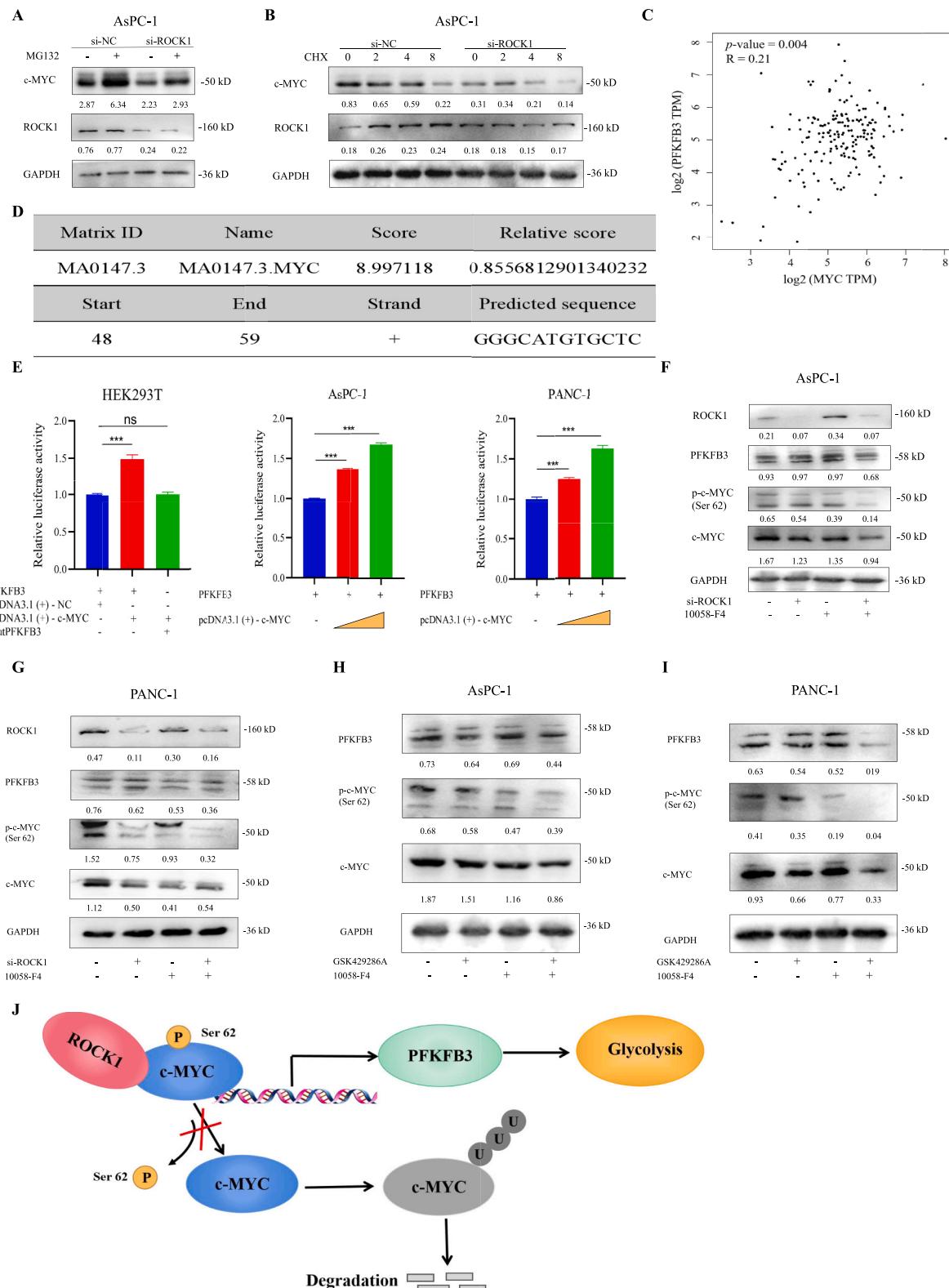


Fig. 5. ROCK1 promotes glycolysis in pancreatic cancer cells through the c-MYC/PFKFB3 signaling axis. **A, B** The stabilizing effect of ROCK1 on the c-MYC protein was detected in MG132- or CHX-treated AsPC-1 cells ($n = 3$). **C** The correlation between c-MYC and PFKFB3 was analyzed using the GEPIA database. **D** The JASPAR and NCBI databases were used to predict the binding sites of c-MYC on the promoter region of the PFKFB3 gene. **E** The binding of c-MYC to the PFKFB3 gene promoter was verified by performing a luciferase reporter assay ($n = 4$). **F–I** Effects of ROCK1 silencing or treatment with a ROCK1 kinase inhibitor combined with a c-MYC inhibitor on PFKFB3 protein expression in AsPC-1 and PANC-1 cells ($n = 3$). **J** Diagram showing the mechanism of action of ROCK1, c-MYC and PFKFB3. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

cells were treated with the c-MYC inhibitor 10,058-F4 after ROCK1 was silenced or treated with the ROCK1 inhibitor GSK429286A to further explain the relationships among ROCK1, c-MYC and PFKFB3. Treatment with 10,058-F4 alone did not seem to effectively inhibit PFKFB3 protein expression. However, when ROCK silencing or ROCK1 inhibitor treatment was combined with 10,058-F4 treatment, the inhibitory effect on PFKFB3 expression was excellent and much greater than the effects of the individual treatments (Fig. 5F-I). Collectively, these results suggest that ROCK1 binds to the c-MYC protein in the cytoplasm and promotes the phosphorylation of c-MYC (Ser 62), thereby enhancing the stability of the c-MYC protein and inhibiting its degradation. C-MYC can bind to the promoter of the PFKFB3 gene to promote its mRNA transcription and subsequent protein expression, thus promoting tumor glycolysis (Fig. 5J).

3.6. AsPC-1 tumor growth is effectively inhibited and GEM sensitivity is increased in nude mice after ROCK1 silencing in nude mice

The silencing efficiency of shRNA was first verified by Western blotting to investigate whether ROCK1 also has carcinogenic activity *in vivo*. ROCK1 protein expression was significantly inhibited after transduction with the shROCK1 lentiviral plasmid, indicating successful transduction of the shROCK1 silencing plasmid. We then investigated whether ROCK1 deficiency enhances the sensitivity of pancreatic cancer cells to GEM. AsPC-1 cells were transduced with shROCK1 and shNC lentiviral plasmids, plated and cultured with a concentration gradient of GEM (0, 1, 2.5, 5, or 7.5 μ M) for 48 h. Cytotoxicity was then detected using an MTT assay. Silencing ROCK1 significantly increased the sensitivity of AsPC-1 cells to GEM (Fig. 6A). Then, the xenograft tumors in the nude mice were analyzed. AsPC-1 cells transduced with shNC and shROCK1 were inoculated into nude mice (approximately 5 weeks old) under the left axilla, and the mice were weighed every four days. On day 44, the mean tumor volume was 181.2 mm³ in the shROCK1 group and 281.9 mm³ in the shNC group. Thus, silencing ROCK1 significantly suppressed the increase in tumor volume in nude mice ($p < 0.05$) compared with that in the shNC group (Fig. 6B-D). These data suggest that silencing ROCK1 can effectively inhibit tumor growth in tumor-bearing mice. *In vitro* experiments have shown that the protein levels of ROCK1, p-c-MYC (Ser 62), and PFKFB3 are positively correlated; thus, we aimed to further validate these results through *in vivo* experiments. Immunohistochemical analysis of different tumor tissues revealed that the p-c-MYC (Ser 62) and PFKFB3 protein levels were also relatively low in tissue samples with low ROCK1 expression. Conversely, tumor tissues with high ROCK1 expression also exhibited increased levels of the p-c-MYC (Ser 62) and PFKFB3 proteins (Fig. 6E), consistent with the results of the *in vitro* experiments. Then, GEM sensitivity was analyzed *in vivo*. Using the subcutaneous xenograft method described above, GEM (25 mg/kg, 50 mg/kg) was injected intraperitoneally every Monday and Thursday beginning when subcutaneous tumors began to form in nude mice on the 20th day. The nude mice were sacrificed after 44 days, and the xenograft tumors were removed. The tumor volume in the shROCK1 group was significantly lower than that in the shNC group, indicating that the sensitivity of xenograft tumors to GEM (** $p < 0.001$) was significantly increased after ROCK1 silencing (Fig. 6F). *In vitro* experiments showed that silencing ROCK1 reduced glycolysis in pancreatic cancer cells, and *in vivo* experiments also verified the effect of ROCK1 knockdown on tumor glycolysis. After ROCK1 silencing, the lactate release rate of pancreatic cancer tumors was significantly reduced (Fig. 6G), and the lactate release rate of tumor tissue decreased by 60.4%.

4. Discussion

Recent studies have shown that ROCK dysregulation is involved in the metastasis and progression of various malignant tumors. ROCK1 shows high kinase activity and protein expression levels during breast

cancer metastasis and advanced breast cancer development, and a similar phenomenon has been observed in colorectal cancer [27–30]. However, the relationship between ROCK1 expression and PC activity is unclear. In this study, we showed that ROCK1 enhances the stability of c-MYC by promoting its phosphorylation (at Ser 62) and inhibiting its degradation via the ubiquitin-proteasome pathway, thus promoting the transcription of the key downstream glycolytic enzyme PFKFB3 at the mRNA level and subsequently increasing its protein expression to increase glycolytic activity in PC cells. These results provide a theoretical basis for identifying important PC treatment strategies.

Our study revealed that ROCK1 was highly expressed in PC and associated with a poor patient prognosis. The GEPIA database and immunohistochemical analyses showed abnormally high ROCK1 expression in the tissues of PC patients. High ROCK1 expression was significantly negatively correlated with patient prognosis, indicating that ROCK1 is a key oncogenic factor in the development of PC. However, the impact of ROCK1 downregulation on the survival rate and prognosis requires validation using clinical data, which needs to be further investigated. The qRT-PCR results further confirmed that the ROCK1 mRNA was highly expressed in AsPC-1 and PANC-1 cells. The other four PC cell lines showed low expression of ROCK1, indicating that ROCK1 expression differed among the cell lines, an effect that may be related to the cell genotype. The genes that regulate this phenomenon may need to be further verified by performing gene mutation or deletion assays. The immunofluorescence results showed that ROCK1 was distributed mainly in the cytoplasm, which is the main site of glycolysis.

We verified the biological function of ROCK1 in the growth of PC cells, by evaluating the effects of changes in ROCK1 expression changes on cell proliferation, migration and apoptosis. ROCK1 silencing significantly inhibited the growth of the two tested PC cell lines and effectively suppressed colony formation. After silencing ROCK1, colony formation by AsPC-1 and PANC-1 cells was significantly inhibited, with inhibition rates of 51.3% and 15.8%, respectively. The flow cytometry results showed that ROCK1 silencing promoted the apoptosis of AsPC-1 and PANC-1 cells. The Transwell assay indicated that silencing ROCK1 significantly inhibited the migration of PC cells, with inhibition rates of 61.6% and 37.9% in AsPC-1 and PANC-1 cells, respectively. These results suggest that ROCK1 plays a role as an oncogenic factor in the development and progression of PC.

Furthermore, the mechanism by which ROCK1 promotes PC cell activity was explored. Since cancer cells utilize glycolysis as the main energy source for rapid proliferation even under oxygen-rich conditions, the cytoplasm is the main site of glycolysis [31], and we speculated that ROCK1 may play a role as an oncogenic factor by affecting glycolysis in PC cells. Glucose uptake and lactic acid release assays showed that ROCK1 silencing significantly inhibited glucose uptake and lactic acid release in the two tested PC cell lines. ECAR analysis showed that ROCK1 silencing significantly inhibited extracellular acidification rate in both PC cell lines, indicating that ROCK1 can promote glycolytic activity in PC cells. The mRNA expression of glycolysis-related genes after ROCK1 silencing was further evaluated, and PFKFB3 and c-MYC mRNA expression was obviously downregulated in both tested PC cell lines. Studies have shown that cancer cells adapt to stressful conditions and continue to proliferate rapidly, in part because the actions of PFKFB3 are diverse and reversible [32–35]. Moreover, the c-MYC protein has been reported to be involved in the regulation of tumor cell growth, cell cycle progression, metabolism, and angiogenesis [36,37]. Western blot analysis revealed that both silencing ROCK1 and treatment with the ROCK1 inhibitor GSK429286A significantly reduced the p-c-MYC (Ser 62) and PFKFB3 protein levels, while ROCK1 did not directly affect the kinase activity of PFKFB3. The co-IP results showed that ROCK1 directly bound to c-MYC but not to PFKFB3. Immunofluorescence experiments also confirmed that ROCK1 and c-MYC were obviously colocalized in the cytoplasm. Subsequently, treatment of AsPC-1 cells with MG132 and CHX showed that ROCK1 enhanced the stability of c-MYC by promoting its phosphorylation (at Ser 62) and inhibiting its degradation. In

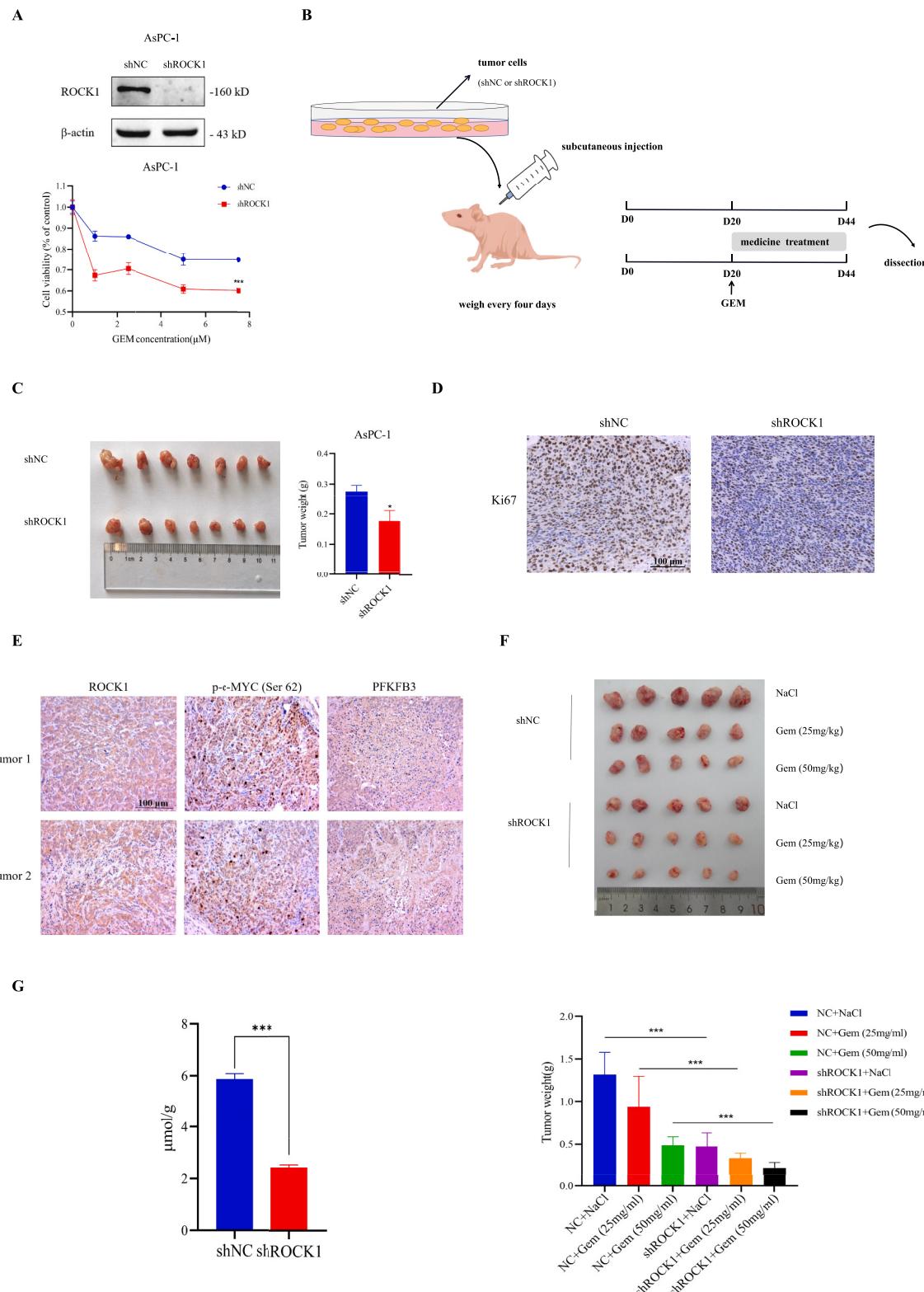


Fig. 6. AsPC-1 tumor growth is effectively inhibited and GEM sensitivity is increased in nude mice after ROCK1 silencing in nude mice. **A** The effect of silencing ROCK1 with an shRNA lentivirus plasmid was verified by Western blotting ($n = 3$). MTT assays were used to detect the viability of shNC/shROCK1 AsPC-1 cells treated with different concentrations or GEM for 48 h. The IC₅₀ values are shown as indicated. (**p < 0.001). **B** Image of tumor-bearing mice. **C** Image of tumors and quantified tumor volume ($n = 7$, *p < 0.05). **D** Representative images of IHC staining showing the protein levels of Ki67 in tumor tissues. Scale bar: 100 μ m. **p < 0.01. **E** Representative images of IHC staining showing the protein levels of ROCK1, p-c-MYC (Ser 62) and PFKFB3 in tumor tissues with high and low ROCK1 levels. Scale bar: 100 μ m. *p < 0.05. **F** Image of tumors and quantified tumor volumes ($n = 5$, ***p < 0.001). **G** Quantification of lactic acid release in tumors ($n = 4$, ***p < 0.001).

addition, the luciferase reporter assay showed that c-MYC promoted the transcription of the PFKFB3 gene in a dose-dependent manner. These results indicate that the ROCK1 protein promotes glycolytic activity in PC cells through the c-MYC/PFKFB3 signaling axis, thereby promoting PC tumor growth.

Finally, our findings further verified the role and mechanism of ROCK1 in promoting the growth of PC *in vivo*. By establishing a nude mouse xenograft model of AsPC-1 cells, we found that ROCK1 silencing significantly reduced the tumor volume and weight in nude mice. Immunohistochemical analysis of tumor tissues revealed that the p-c-MYC (Ser 62) and PFKFB3 protein levels were also relatively high in tumor tissues with high ROCK1 expression and low in tumor tissues with low ROCK1 expression. These results indicated that ROCK1 promoted the growth of PC *in vivo*, and the positive correlations of ROCK1 expression with p-c-MYC (Ser 62) and PFKFB3 expression were consistent with the results *in vitro*. In our study, when combined with the pancreatic cancer chemotherapy drug gemcitabine (GEM), ROCK1 silencing significantly increased the sensitivity of pancreatic cancer cells to gemcitabine both *in vivo* and *in vitro*. We hope to provide new ideas for clinical research and chemotherapy resistance in pancreatic cancer by identifying new targets and new inhibitors. In addition, ROCK1 silencing resulted in a decrease in lactate content in tumor tissue, indicating that knocking down ROCK1 inhibits glycolysis at the animal level. However, the relationships between the levels of the ROCK1, p-c-MYC (Ser 62) and PFKFB3 proteins were verified only by immunohistochemistry at the animal level; these relationships will be the focus of subsequent research. Moreover, the mechanism by which ROCK1 affects enzymatic activity in the ubiquitin-proteasome degradation pathway to stabilize c-MYC needs to be further study.

5. Conclusions

In conclusion, our findings reveal that ROCK1 acts through the c-MYC/PFKFB3 signaling axis to enhance glycolytic activity in PC cells, thereby enhancing their growth. Moreover, knockdown of ROCK1 can increase the sensitivity of PC cells to GEM. This paper indicates that ROCK1 is an effective therapeutic target for PC and that its positive regulation of glycolysis may provide an effective clinical therapeutic strategy for PC.

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Ethical approval and consent to participate

All animal experiments were performed according to protocols approved by the Ethics Committee of China Pharmaceutical University (approval no. SYXK 2021-0011).

Patient consent for publication

Not applicable.

CRediT authorship contribution statement

Shuyang Pang: Writing – original draft, Project administration, Methodology. **Yuting Shen:** Writing – review & editing, Visualization, Data curation. **Yanan Wang:** Writing – review & editing, Visualization, Data curation. **Xuanning Chu:** Writing – review & editing, Formal analysis, Data curation. **Lingman Ma:** Supervision, Funding acquisition. **Yiran Zhou:** Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

The data and materials are available from the corresponding author upon request.

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