Suppressive effect of YY1-mediated RGS22 regulation on the proliferation, migration and invasion of pancreatic ductal adenocarcinoma

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Abstract. Regulator of G-protein signaling 22 (RGS22) is specifically expressed in the testis and in tumors of epithelial origin, but the expression and role of RGS22 in pancreatic cancer are unclear. In this study, 52 pairs of pancreatic ductal adenocarcinoma (PDAC) and adjacent non-neoplastic tissue samples with the corresponding clinical data were used to examine the expression of RGS22 and its relationship with PDAC prognosis. The findings showed that the expression of RGS22 was higher in the PDAC tissues than in the adjacent non-tumorous tissues and its expression was associated with the degree of blood vessel invasion. The *in vitro* experiments with PDAC cell lines and a normal control cell line showed that the proliferation, invasion, and metastasis of PDAC cells were suppressed by RGS22 overexpression and enhanced by RGS22 knockdown. The *in vivo* effect of RGS22 on PDAC xenografts was studied using subcutaneous implantation of tumor cells in BALB/cA-nu mice, and the results corroborated the *in vitro* findings. Analysis of the regulators of RGS22 showed that it was positively regulated by the transcription factor Yin Yang-1 (YY1). Thus, YY1-mediated RGS22 regulation suppressed the proliferation, migration, and invasion of PDAC.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of tumor-related death worldwide, with a survival time of only ~6 months and a 5-year survival rate of <8% (1,2). The poor outcome prospects are partially attributable to its insidious onset. More than 80% of patients have metastatic or locally advanced PDAC at first diagnosis (2,3). In addition, PDAC is not sensitive to radiotherapy or chemotherapy (4-9).

Yin Yang-1 (YY1) is a widely expressed transcription factor that belongs to the GLI-Kruppel class of zinc finger proteins. As its name suggests, YY1 can positively or negatively control genes depending on the DNA-binding sites or cell types (6-9). YY1 is highly expressed in PDAC where it acts as a tumor suppressor (10). According to our previous findings, YY1 can inhibit the proliferation, migration, and invasion of PDAC by regulating the expression of different downstream molecules (10,11).

Regulator of G-protein signaling 22 (RGS22) is a newly identified protein that belongs to the RGS family, which negatively regulate heterotrimeric G-protein signaling (12-15). RGS22 is specifically expressed in the testis and in cancers of epithelial origin (16). Furthermore, overexpression of RGS22 can inhibit invasion and migration in EC9706 esophageal squamous cell carcinoma cells (17). In PDAC, RGS22 acts as a tumor suppressor by repressing the migration of PDAC cells via coupling to GNA12/13 to inhibit stress fiber formation (18). However, the expression pattern and regulation of RGS22 in PDAC, and its influence on tumor cell proliferation still requires investigation. Our previous ChIP-Sequencing (ChIP-Seq) results suggested that YY1 could directly bind to the promoter region of RGS22 (10) (Table SI), indicating that YY1 may regulate the transcription of RGS22. Therefore, the aim of the present study was to investigate the expression and regulation of RGS22 in PDAC tissues, and the potential role of YY1 in the regulation of RGS22 and its inhibitory effects on PDAC.

Materials and methods

Patients and pancreatic tissues. A total of 52 pairs of tumorous and adjacent nontumorous human pancreatic tissue samples (between February 2015 and August 2016) were collected from patients who underwent pancreaticoduodenectomy for PDAC at the first Affiliated Hospital of Nanjing Medical University. The patients were aged between 44 and 91 years, with a median age of 52 years, and included 36 male and 16 female patients. Each tissue sample was divided into two parts after surgical resection. One part of the tissue was fixed in 5% formalin and embedded in paraffin after 24 h for immunohistochemistry (IHC). The other part of the tissue was stored in liquid nitrogen for subsequent RNA extraction. Pathological analysis of all the tissue samples was performed by two experienced pathologists, who confirmed that the patients had PDAC based on the pathological features of the samples. Patients were followed up regularly until November 1st, 2018. None of the patients had received radiation therapy or chemotherapy before cancer resection. TNM staging was performed based on the 8th edition of the American Joint Committee on Cancer guidelines (19). Patients were given written notice before the surgery was performed, and the study was approved by the Ethics Committees of the first Affiliated Hospital of Nanjing Medical University. Informed consent was obtained from the participants or their legal guardians. In addition, TCGA and GTEX databases (http://gepia.cancer-pku. cn/index.html) were used to analyze the mRNA expression of RGS22 in pancreatic cancer/adjacent normal tissues.

Cell lines and culture. PDAC cell lines (PANC-1, CFPAC-1, BXPC-3, and MiaPaCa-2) and a normal human pancreatic ductal cell line (hTERT-HPNE, also known as HPNE) were obtained from Shanghai Cell Bank. Cells were cultured in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Wisent Inc.) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.). Cell lines underwent routine testing for mycoplasma contamination every 3 months. The genetic identity of the cell lines was confirmed by short tandem repeat profiling.

Preparation of stable cell lines. PANC-1 cells stably overexpressing YY1 were constructed as described previously (10). YY1-knockdown lentiviruses (lentiviral plasmid: pLKD-CMV-G&PR-U6-YY1 shRNA) were constructed by Sunbio Medical Biotechnology Co., Ltd. as described previously (10). RGS22-overexpression (lentiviral plasmid: pSLenti-EF1a-mCherry-P2A-Puro-CMV-RGS22-3Flag) and RGS22-knockdown lentiviruses (lentiviral plasmid: pLKD-CMV-G&PR-U6-RGS22 shRNA) were constructed by Heyuan Biotechnology Co., Ltd. The sequences of YY1-shRNA, RGS22-shRNA, and scramble shRNA are shown in Table SII. When PANC-1 and CFPAC-1 cells reached ~40% confluence they were infected with RGS22-overexpression, RGS22-knockdown, YY1-knockdown, or the respective control lentiviruses. The multiplicity of infection (MOI) used to infect PANC-1 and CFPAC-1 cells was 10. Polybrene (final concentration, 5 µg/ml) was added to the cells before infection with lentiviruses. After 12-20 h of infection, the medium was replaced with fresh medium. After 72 h of infection, puromycin (final concentration, 5 µg/ml; MilliporeSigma) was added and the medium was replaced every 2-3 days. Stable cell lines (PANC-1-Vector, PANC-1-RGS22, CFPAC-1-Scramble shRNA, CFPAC-1-RGS22-shRNA and CFPAC-1-YY1-shRNA) were selected by culturing for 14 days in media containing 5 µg/ml puromycin. The puromycin concentration used for maintenance was 5 µg/ml. RGS22 and YY1 expression was confirmed by reverse transcription-quantitative PCR (RT-qPCR) and western blotting.

Transient transfection of siRNAs. Three different sequences of RGS22-knockdown siRNAs were obtained: RS1 sense, 5'-GCU UGCAACCUCUCACAAATT-3' and antisense, 5'-UUUGUG AGAGGUUGCAAGCTT-3'; RS2 sense, 5'-GCUUAUCAC AACCUCCUAATT-3' and antisense, 5'-UUAGGAGGUUGU GAUAAGCTT-3'; RS3 sense, 5'-GCACCAAGAUUCUGUGUU ATT-3' and antisense, 5'-UAACACAGAAUCUUGGUGCTT-3'); and scrambled siRNA negative control sense 5'-UUCUCC GAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACAC GUUCGGAGAATT-3'. siRNAs were synthesized by Shanghai GenePharma Co., Ltd. These RGS22-knockdown siRNAs (5 µg/ml) and 5 µg/ml Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.) were transfected into wild-type PANC-1 cells for 72 h at 37°C according to the manufacturer's instructions. At 72 h after transfection, the expression of RGS22 was determined by western blotting to verify transfection efficiency. RS3 was chosen to construct RGS22-knockdown lentiviruses, as it exhibited the maximum efficiency out of the three sequences assessed (Fig. S1). CCK-8, colony formation, EdU, wound healing assays and Transwell assays were performed 72 h after RS3 transfection.

RNA isolation and RT-qPCR. TRIzol® reagent (Thermo Fisher Scientific, Inc.) was used to extract total RNA from tissue samples and cells according to the manufacturer's instructions. The RNA was reverse transcribed to cDNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc.). RT-qPCR was performed in a Step One Plus Real-time PCR System (Thermo Fisher Scientific, Inc.) using the SYBR green reagent (Thermo Fisher Scientific, Inc.). Each quantitative round of PCR was performed in triplicate and repeated independently three times. The β-actin gene was used for normalization. The following primers constructed by Nanjing Realgene Biotechnology Co., Ltd. were used: RGS22 forward, 5'-AACTGGAGTTTGAAC ATTTCCG-3' and reverse, RGS22, 5'-GCCTTCCTTTGATTT CGATCTC-3'; YY1 forward, 5'-ACGGCTTCGAGGATCAGA TTC-3' and reverse, 5'-TGACCAGCGTTTGTTCAATGT-3'; and β-actin forward, 5'-CTCCATCCTGGCCTCGCTGT-3' and reverse, 5'-GCTGTCACCTTCACCGTTCC-3'.

Western blotting. Proteins were extracted from PDAC cells using RIPA lysis buffer (Abcam). Protein concentrations were measured using a DC protein assay kit (Bio-Rad Laboratories, Inc.). Equal amounts of proteins (10 µg/lane) were loaded on a 10% SDS-gel, resolved using SDS-PAGE, and transferred to a PVDF membrane. Nonspecific protein interactions were blocked by incubation in 5% nonfat dry milk in TBS with 0.1% Tween 20 (TBST) buffer at room temperature for 1 h and then washed with TBST. Membranes were then incubated at 4°C overnight with primary antibodies in fresh blocking buffer.

Anti-β actin (cat. no. ab8226; dilution, 1:1,000), anti-YY1 (cat. no. ab109228; dilution, 1:1,000), and anti-RGS22 (cat. no. ab131048 and ab248357; dilution, 1:1,000) antibodies were purchased from Abcam. The blots were then washed and incubated with HRP-conjugated secondary antibodies (cat. no. A0208; dilution, 1:1,000; Beyotime Institute of Biotechnology) for 1 h at room temperature. Each western blotting experiment was repeated three times independently.

Tissue microarrays and IHC. Tissue microarrays containing 52 pairs of PDAC samples, and their corresponding non-tumorous tissues were constructed, and IHC was performed by Wuhan Servicebio Technology Co., Ltd. The expression levels of RGS22 were assessed based on the staining intensity and the percentage of positively stained cells. Expression levels were evaluated according to the staining intensity (0, absent; 1, weak; 2, moderate; and 3, strong staining) and proportion of positive cells (0, <10%; 1, 10 to <50%; and 2, ≥50% of cells). The IHC score was calculated using the following formula: IHC score=positive staining score x staining intensity score.

Cell counting kit-8 (CCK-8) assay. A CCK-8 assay (Dojindo Molecular Technologies, Inc.) was used to assess cell proliferation. A total of 2,000 cells per well were cultured in 96-well plates for 5 days. At the same time of each day, 10 μl CCK-8 reagent was mixed with 90 μl DMEM and added to each well. After 2 h of incubation, the absorbance of each well was assessed at 450 nm using a spectrophotometer.

Colony formation assay. PDAC cells were plated at a concentration of 500 cells per well in 6-well plates and cultured in supplemented medium for 20 days. Colonies were stained with a 0.05% crystal violet solution for 30 min at room temperature. Photos of the colonies were taken using a Zeiss microscope. The number of colonies consisting of >50 cells were counted using a light microscope (magnification, x100). Each sample consisted of three duplicate wells, with three repeats.

5-EdU assay. A 5-EdU assay was performed using an EdU kit (Guangzhou RiboBio, Ltd.) according to the manufacturer's instructions. Each sample consisted of three duplicate wells and was repeated three times independently.

Wound-healing assay. A wound-healing assay was performed to evaluate cell migration. Briefly, cells were seeded at a density of $1x10^3$ cells/well into six-well plates. When cells in the 6-well plates formed a confluent monolayer, the monolayer was scratched with a sterile 200-μl pipette tip. Images were taken using an inverted fluorescence microscope (magnification, x100; Olympus Corporation) immediately and 48 h after scratching. The relative wound areas were then measured using ImageJ 1.8.0 (National Institutes of Health).

Transwell assays. To assess the migratory and invasive abilities of the cells, Transwell assays were performed. For the migration assays, $4x10^3$ cells were suspended in 1 ml serum-free medium and then added to the upper chamber, and 1 ml supplemented medium was added to the bottom chamber. After 24 h of culturing, the cells in the upper chamber were wiped away using a cotton swab, and the chambers were stained with 1%

crystal violet solution for 20 min at room temperature. For the invasion experiments, Matrigel (BD Bioscience Pharmingen) was first added to the upper chamber and allowed to solidify, after which the above steps were performed, except cells were cultured for 48 h. The chambers were imaged using an inverted fluorescence microscope (magnification, x100; Olympus Corporation). All the experiments were repeated three times.

Luciferase activity assay. The results of our previous ChIP-Seq analysis (10) predicted the key YY1-binding site of RGS22 (5'-GAAAAACCATTAAAAAGTTT-3'). Luciferase activity assays were performed based on this result by Heyuan Biotechnology Co., Ltd. A luciferase reporter construct containing the human RGS22 promoter (-1,331/151, upstream and downstream of translation start site, Supplementary Materials and methods) was prepared using the pGL4.10-basic vector (Promega Corporation). Heyuan Biotechnology Co., Ltd. synthesized a DNA fragment of the RGS22 promoter region (including the sites of restriction enzymes). The DNA fragment was subcloned into the pGL4.10-basic vector to construct pGL4.10-RGS22-promoter (WT) (WT pRGS22) recombinant plasmid and then confirmed by sequencing. The mutant construct containing the RGS22 promoter in which the presumed YY1-binding site (GAAAAACCATTAAAAAGTTT, nucleotides -554 to -573), was deleted was also constructed. The construct was named pGL4.10-RGS22-promoter (MT pRGS22).

Transfections were performed using Lipofectamine® 2000, according to the manufacturer's protocol. YY1 overexpression PANC-1 cells or control cells were plated into 12-well cell culture plates ($2x10^5$/well) 1 day before transfection. Each transfection was performed using 1 μg luciferase reporter construct (WT pRGS22 or MT pRGS22) plus 2.5 ng Renilla luciferase reporter vector, pRL-SV40 as an internal control (Promega Corporation). A total of 48 h after transfection, cells were washed with PBS and lysed using 1x lysis buffer. Firefly and Renilla luciferase activities were measured using a GloMax-20/20 luminometer (Promega Corporation) using a Dual-Luciferase Reporter Assay System (Promega Corporation). Firefly luciferase activity was normalized to the Renilla luciferase activity. Each experiment was performed in triplicate and independently repeated three times.

Nuclear protein extraction and electrophoretic mobility shift assay (EMSA). Nuclear protein extraction was performed using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. EMSA was performed using the DIG Gel Shift Kit (Roche Diagnostics GmbH) according to the manufacturer's instructions. Probes (wild-type probe, 5'-GAAAAACCATTA AAAAGTTT-3'; mutant probe, 5'-GAAAACTTCCTAAAA AGTTT-3') for this experiment were synthesized by Heyuan Biotechnology Co., Ltd.

Construction of the in vivo model. For the construction of *in vivo* models of PDAC, 12 4-week-old female, nude mice (BALB/cA-nu) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The mice were randomly divided into two groups (PANC-1-Vector and PANC-1-RGS22), and $1x10^6$ PANC-1-Vector or PANC-1-RGS22 cells were injected into the abdominal cavity of each mouse. The size of the neoplasms
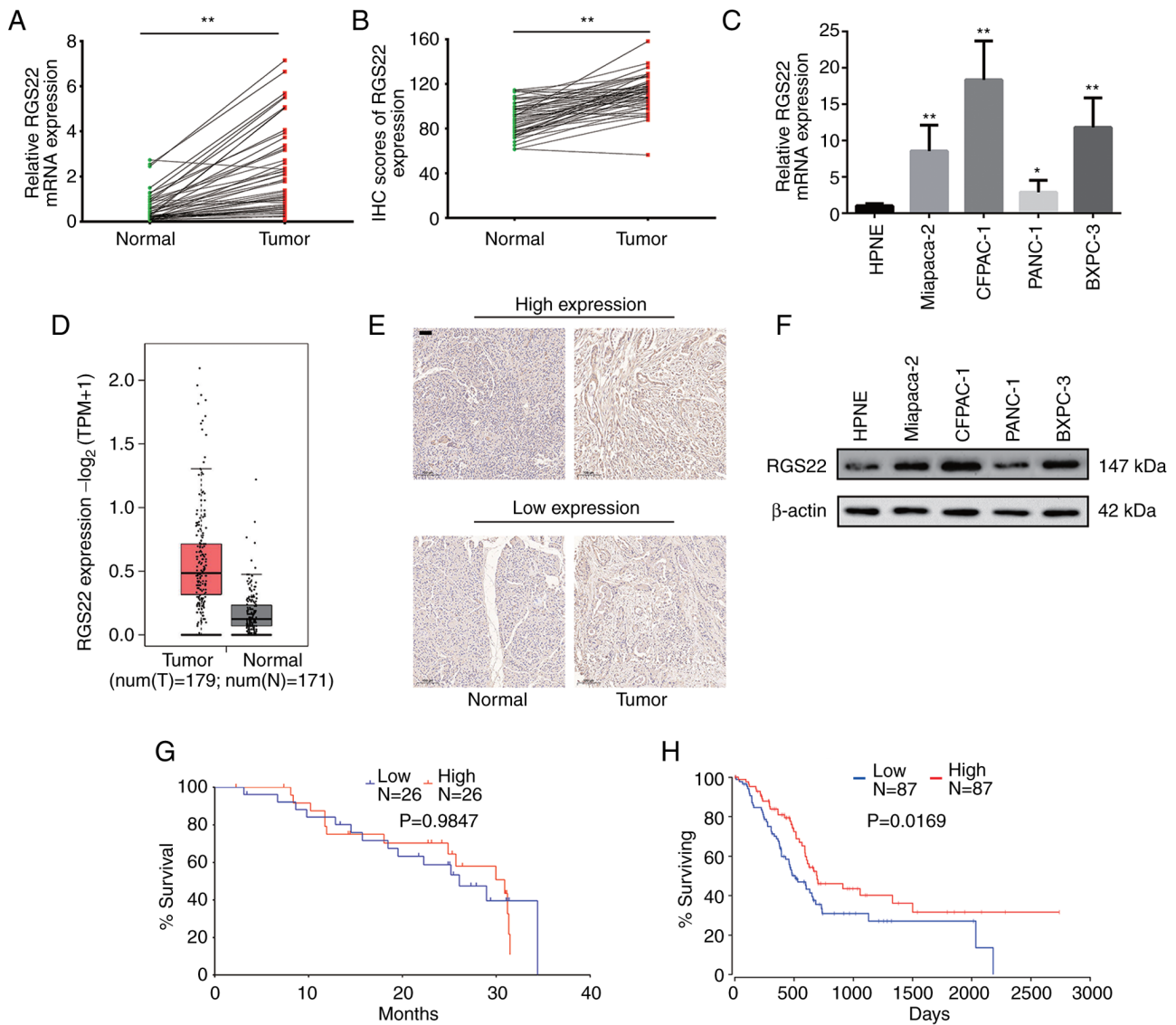
Figure 1. Expression of RGS22 in PDAC. (A) Boxplot showing RGS22 RNA expression in 52 pairs of PDAC tissues and adjacent tissues, as determined by RT-qPCR. (B) A boxplot showing RGS22 protein expression in 52 pairs of PDAC tissues and adjacent tissues, as determined by IHC. (C) RT-qPCR analysis of RGS22 RNA expression in a panel of human PDAC cell lines and the HPNE cell line. (D) A boxplot showing RGS22 RNA expression in 179 PDAC tissues and 171 normal pancreatic tissues [data from TCGA and GTEx; unit of measurement: $\log_2$(TPM+1)]. (E) RGS22 expression was lower in the PDAC cells than in the adjacent tissues, as determined by IHC. Scale bar, 50 μm. (F) Western blot analysis of RGS22 protein expression in a panel of human PDAC cell lines and the HPNE cell line. (G) Kaplan-Meier curves for OS based on RGS22 expression in 52 cases of PDAC. (H) Kaplan-Meier curves for OS according to RGS22 expression in 174 cases of PDAC (data from TCGA and GTEx). *$P \leq 0.05$, **$P \leq 0.01$. RGS22, Regulator of G-protein signaling 22; PDAC, pancreatic ductal adenocarcinoma; RT-qPCR, reverse transcription-quantitative PCR; IHC, immunohistochemistry; TCGA, The Cancer Genome Atlas; GTEx, Genotype-Tissue Expression; TPM, transcripts per million; OS, overall survival.

was measured every 6 days for 30 days, and the formula (width^2 x length)/2 was used to calculate the tumor volumes. The mice were anesthetized by intraperitoneal injection of 5% chloral hydrate (the dose of chloral hydrate was 400 mg/kg body weight). The tumors were excised after the mice were anesthetized and sacrificed by cervical dislocation. IHC was performed to determine the Ki-67 levels (1:200; Wuhan Servicebio Technology Co., Ltd.) of the tumor samples. The animal experiments were approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University. All methods were performed in accordance with the relevant guidelines and regulations.

Statistical analysis. Statistical analysis was performed using SPSS version 22.0 (IBM Corp.) and GraphPad Prism version 6.0 (GraphPad Software, Inc.). Quantitative data are presented as the mean ± SD. The association between RGS22 expression with clinicopathological features was analyzed using a Pearson's χ^2 test. The Kaplan-Meier test was employed to calculate the survival rates of the two groups. A Student's t-test was used to analyze differences in the mean values between two groups. Area calculations were performed using ImageJ. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of RGS22 in PDAC samples. RT-qPCR was used to examine the mRNA expression levels of RGS22 in 52 pairs

of PDAC tissue samples and adjacent non-neoplastic tissue samples. RGS22 expression was upregulated in the PDAC tissues, and its expression was significantly higher than that in the adjacent non-neoplastic tissues (P<0.0001, Fig. 1A). Similar findings were obtained with IHC analysis of RGS22 protein expression, and expression differed significantly between the PDAC and non-neoplastic tissues (P<0.0001, Fig. 1B and E). These results are consistent with data from TCGA and GTEx databases (Fig. 1D) (20).

The expression of RGS22 was also assessed using western blotting and RT-qPCR in four PDAC cell lines (PANC-1, CFPAC-1, BxPC-3, and MiaPaCa-2) and a normal human pancreatic ductal cell line (HPNE). Compared with the HPNE cells, the PDAC cells exhibited higher expression of RGS22 (Fig. 1C and F). PANC-1 (which had relatively low expression of RGS22) and CFPAC-1 (which had relatively high expression of RGS2) cells were used for all subsequent experiments.

Correlation between RGS22 expression and the prognosis of PDAC. Survival analysis included 52 patients. The cutoff value for low/high RGS22 expression was determined by the median expression value based on RT-qPCR data. There was no statistically significant difference in overall survival between the low RGS22 and high RGS22 groups (Fig. 1G). However, data from TCGA and GTEx databases showed that patients with higher RGS22 expression had significantly better overall survival (Fig. 1H, P=0.0169) (20). In addition, the correlation between RGS22 expression and clinical characteristics of the PDAC patients was analyzed. As shown in Table I, the expression of RGS22 was associated with blood vessel invasion (P=0.0438); that is, patients with high RGSS22 expression had a lower degree of blood vessel invasion.

RGS22 suppresses the proliferation, migration, and invasion of pancreatic cancer cells in vitro. To assess the effect of RGS22 on the development of PDAC, stable RGS22 overexpression or knockdown cells (PANC-1-Vector, PANC-1-RGS22, CFPAC-1-Scramble shRNA, and CFPAC-1-RGS22 shRNA) were constructed using lentiviruses. RT-qPCR and western blotting confirmed the change in levels of RGS22 in these cell lines (Fig. 2A and B). The effects of RGS22 on the proliferation of PDAC cells were investigated by CCK-8, colony-formation, and EdU assays. RGS22 overexpression resulted in a significant decrease in the proliferation of PANC-1 cells compared with the control group (Fig. 2C). Conversely, RGS22 knockdown resulted in a significant increase in the proliferation of CFPAC-1 cells (Fig. 2D). Similar results were observed in the colony formation assays. The number of colonies were significantly lower in RGS22-overexpressing PANC-1 cells and significantly higher in RGS22-knockdown CFPAC-1 cells compared with their respective controls (Fig. 2E and F); there was a substantially large difference in colony formation between the two cell lines (PANC-1 and CFPAC-1) as compared to their growth curves (CCK-8 assay). The difference may be attributed to the duration of each experiment; in colony formation assays, cells were cultured for 20 days, thus the time scale to allow for measurement of large differences was larger than in the CCK-8 experiments in which cells were cultured for only 5 days. The EdU assay (Fig. 2G-J) showed that the proportion of EdU-positive nuclei was significantly

Table I. Association between RGS22 expression and the clinicopathological features of PDAC.

| Variable | RGS22 expression | | P-value |
	High	Low	
Sex			0.0714
Male	21	15	
Female	5	11	
Age, year			0.3965
≤65	14	17	
>65	12	9	
Location			0.3584
Head	17	20	
Body, tail	9	6	
Differentiation			0.4164
Poor	2	5	
Moderate or High	24	21	
Tumor-Node-Metastasis stage			0.7801
I-IIA	11	12	
IIB-IV	15	14	
T stage			0.1259
T1 or T2	21	16	
T3 or T4	5	10	
N stage			0.7814
0	12	13	
1 or 2	14	13	
Blood vessel invasion			0.0438[a]
Absent	13	6	
Present	13	20	
Serum CA19-9, kU/l			0.2111
≤39	5	9	
>39	21	17	

[a]P<0.05.

decreased in RGS22-overexpressing PANC-1 cells and significantly increased in RGS22-knockdown CFPAC-1 cells. Together, these results demonstrate that RGS22 suppresses the proliferation of pancreatic cells *in vitro.*

Wound-healing assays and Transwell assays were performed to investigate the effects of RGS22 on the invasion and migration of PDAC cells. The results of the wound-healing assays showed that RGS22 knockdown resulted in an increase in the rate of wound healing and RGS22 overexpression resulted in a decrease in the rate of wound healing (Fig. 3A-D). Similar results were obtained with the Transwell assays; RGS22 overexpression suppressed the migration and invasion of PANC-1 cells, while RGS22 knockdown promoted the migration and invasion of CFPAF-1 cells (Fig. 3E-H). These results indicate that RGS22 suppresses the migration and invasion of pancreatic cancer cells *in vitro.*
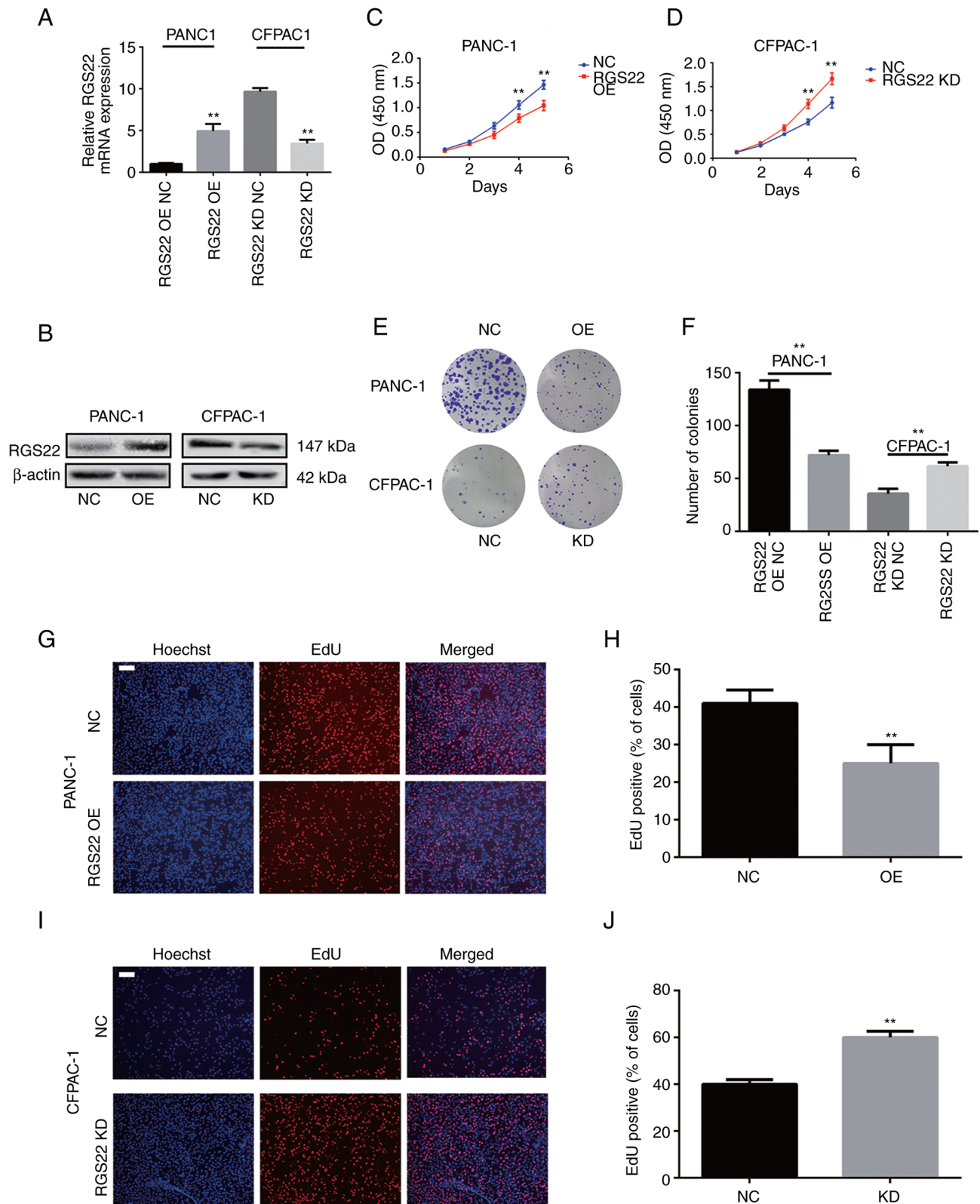
Figure 2. RGS22 suppresses proliferation of pancreatic cancer cells. Relative (A) mRNA and (B) protein expression levels of RGS22 in RGS22-knockdown and RGS22-overexpressing pancreatic cancer cells. (C and D) Cell Counting Kit-8, (E and F) colony formation, and (G-J) EdU assays were performed to analyze the effects of RGS22 knockdown or overexpression on cell proliferation. Magnification, x100; scale bar, 100 μm. Data are presented as the mean ± SD of three independent experiments. **P≤0.01. RGS22, Regulator of G-protein signaling 22; OD, optical density; OE, overexpression; NC, negative control; KD, knockdown.

RGS22 suppresses the growth of PDAC in vivo. To study the *in vivo* effects of RGS22 on PDAC cells, stable RGS22-overexpressing PANC-1 cells were subcutaneously implanted into BALB/cA-nu mice. The size of the xenograft tumors was measured every 6 days with a pair of calipers for 30 days. The size of the tumors formed by the RGS22-overexpressing
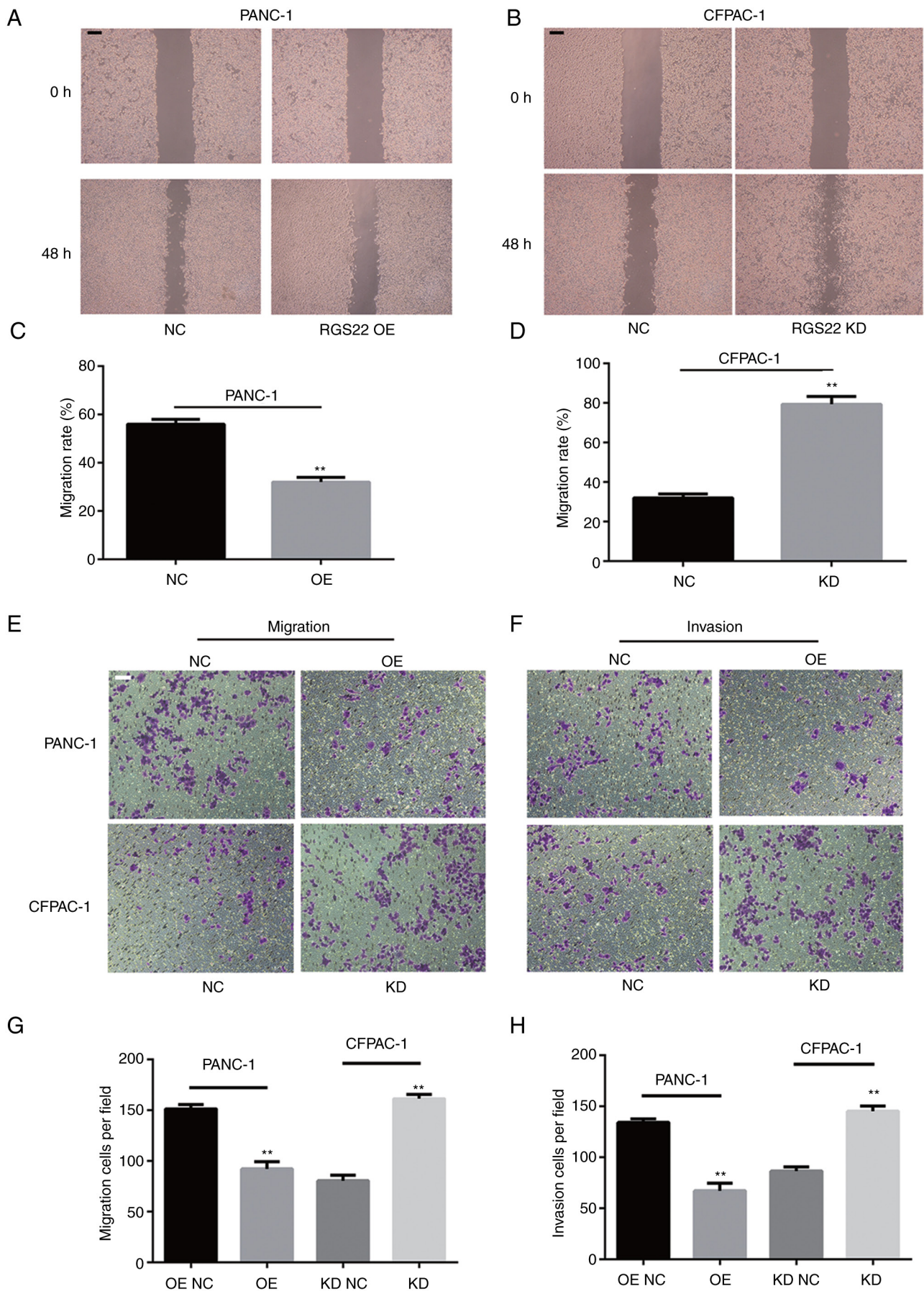
Figure 3. RGS22 suppresses the migration and invasion of pancreatic ductal adenocarcinoma cells. (A-D) Wound-healing assays were performed using the RSG22-overexpressing PANC-1 cells and RGS22-knockdown CFPAC-1 cells. Images were obtained after 0 and 48 h. (E-H) Cell migration and invasion assays were performed in RGS22-overexpressing PANC-1 cells and RSG22-knockdown CFPAC-1 cells. **P≤0.01. RGS22, Regulator of G-protein signaling 22; OE, overexpression; NC, negative control; KD, knockdown.
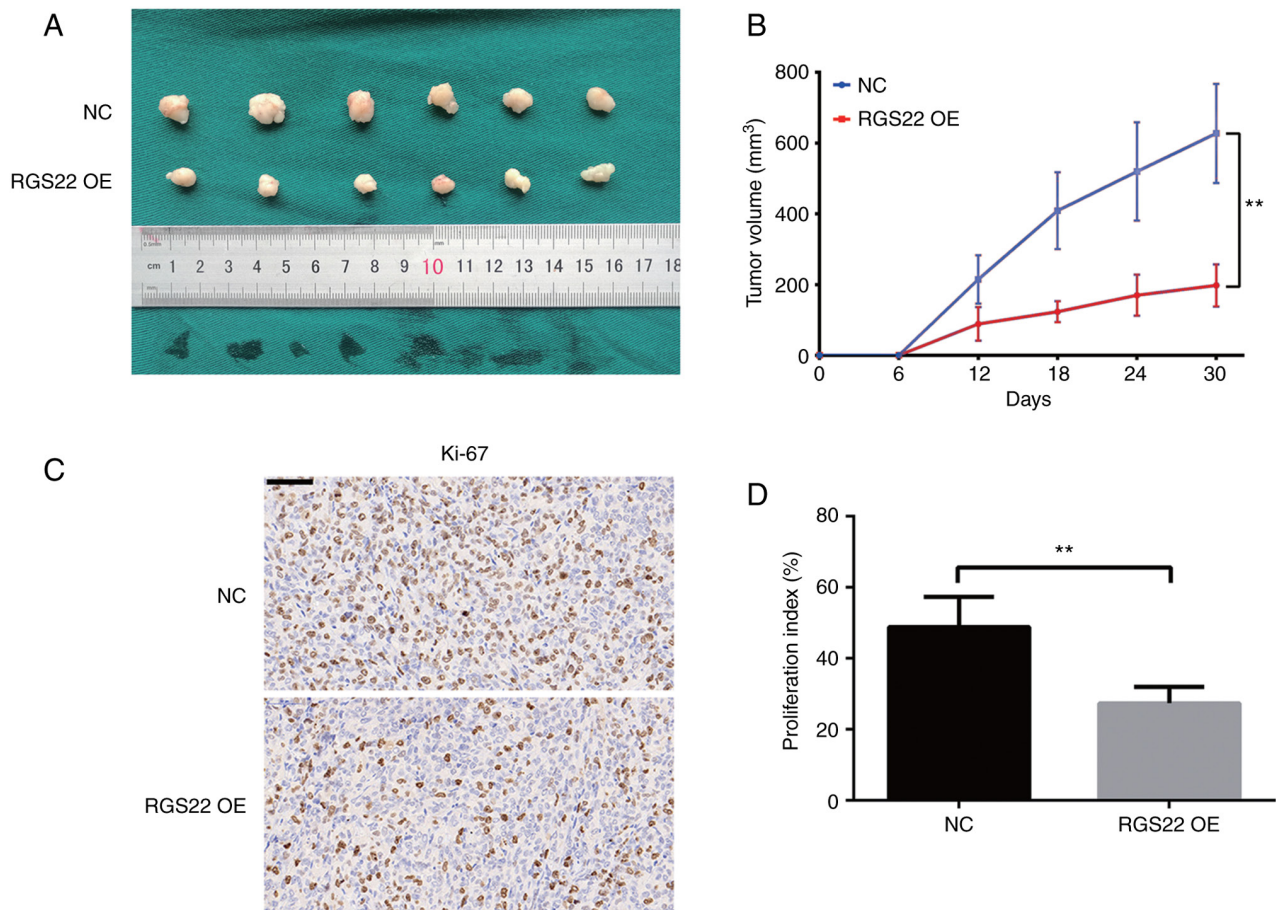
Figure 4. RGS22 suppresses pancreatic ductal adenocarcinoma growth *in vivo*. (A and B) Tumors were obtained from nude mice injected subcutaneously with PANC-1 cells overexpressing RGS22, and the growth curve was determined based on measurements of tumor volume over 30 days. (C and D) Immunohistochemistry analysis of Ki-67 protein expression in subcutaneous tumors from mice injected with RGS22-overexpressing PANC-1 cells. Magnification, x100; scale bar, 100 μm. **P≤0.01. RGS22, Regulator of G-protein signaling 22; OE, overexpression; NC, negative control.

PANC-1 cells was significantly smaller than that of the control group tumors (Fig. 4A and B). In addition, Ki-67 staining showed that RGS22 overexpression decreased the proliferation index of the xenograft tumors (Fig. 4C and D).

Direct regulation of RGS22 by YY1. The results of the western blotting and RT-qPCR experiments showed that RGS22 expression was positively correlated with YY1 expression (Fig. 5A-C). These results indicate that RGS22 may be regulated by the transcription factor YY1. To verify this hypothesis, EMSA and luciferase experiments were performed. Based on the predicted binding site from the previous analysis, a digoxigenin-labeled probe was constructed for the EMSA experiment. The probe bound to YY1 and the YY1 antibody to form a specific super shift (Fig. 5E). These results showed that YY1 directly bound to the promotor region of RGS22.

To further investigate the effect of YY1 on RGS22 expression, luciferase assays were performed. RGS22 reporter gene plasmids and its mutant plasmids were constructed as shown in Fig. 5D. The reporter plasmids were co-transfected with YY1-overexpression plasmids. Overexpression of YY1 significantly reduced the luciferase values compared with those observed in the vector cells (Fig. 5F). In addition, when the putative binding site of YY1 was mutated, the luciferase value decreased; this demonstrates the specificity of the binding

site. These results show that YY1 binds to a specific region of the RGS22 promotor to positively control the expression of RGS22.

RGS22 as a functional target of YY1. Previous studies have shown that YY1 acts as a tumor suppressor in PDAC. To investigate whether the effects of YY1 on pancreatic cells are mediated by RGS22, RGS22 expression was knocked down using a specific siRNA construct in PANC-1 cells stably overexpressing YY1 for the recovery experiments. The results of the CCK-8, colony formation, and EdU assays showed that downregulation of RGS22 in YY1-overexpressing cells restored the inhibition of PANC-1 cell proliferation by YY1 (Fig. 6A-E). Cellular Transwell assays and wound-healing assays illustrated that downregulation of RGS22 restored the inhibitory effect of YY1 overexpression on the invasion and migration of PANC-1 cells (Fig. 6F-J). These results indicate that YY1 inhibits the proliferation, invasion, and migration of PDAC cells by targeting RGS22.

Discussion

The *in vitro* and *in vivo* findings of the present study indicate that RGS22 inhibited tumor proliferation, invasion, and metastasis of PDAC. These findings were corroborated by
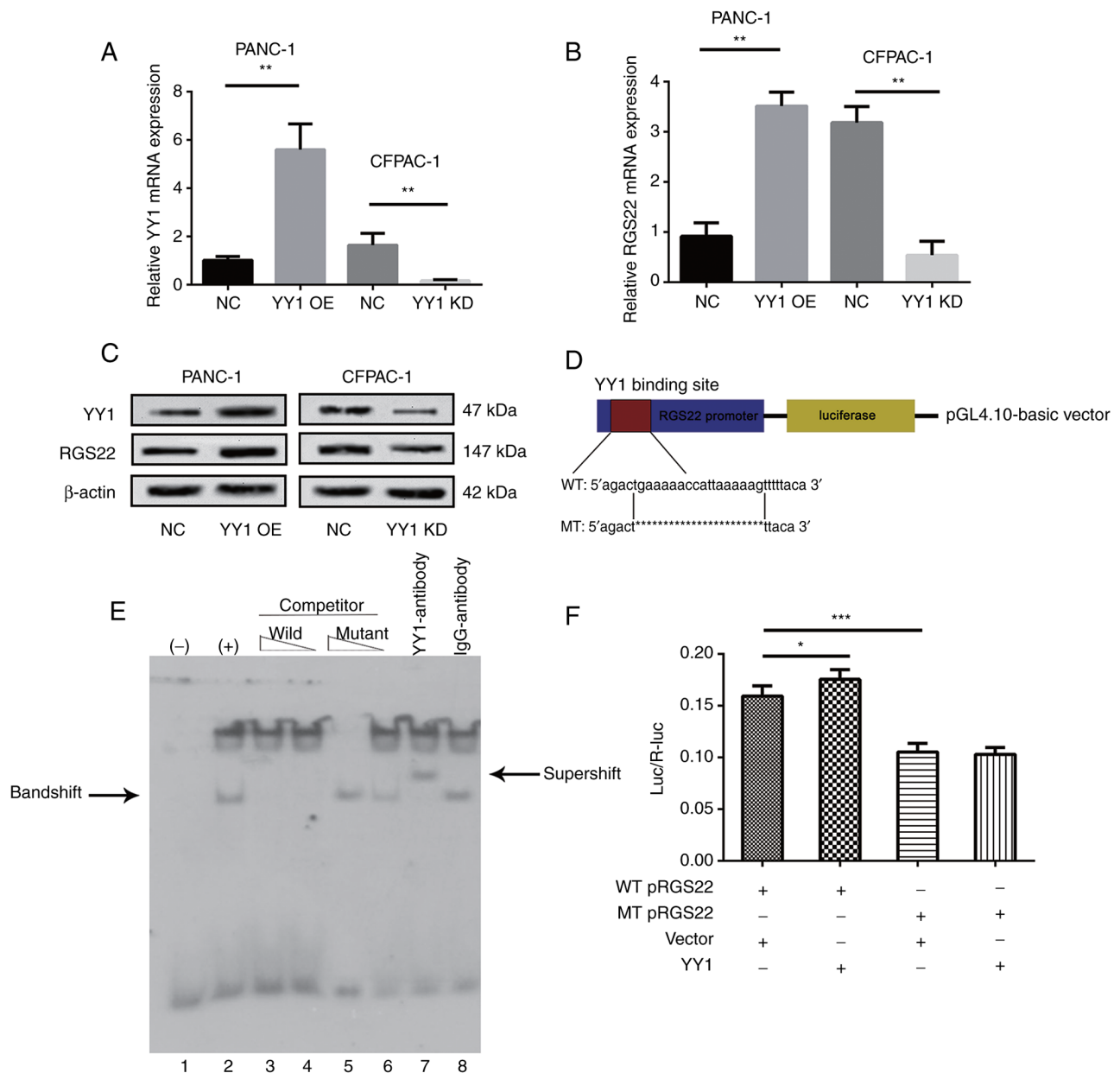
Figure 5. Regulation of RGS22 expression by the binding of YY1 directly to the RGS22 promoter. (A-C) RGS22 expression in YY1-overexpressing PANC-1 cells or YY1-knockdown CFPAC-1 cells was measured by reverse transcription-quantitative PCR and western blotting. (D) Schematic diagram of the luciferase reporter construct containing the human RGS22 promoter and the mutant construct containing the RGS22 promoter in which the predicted YY1 binding site was mutated. (E) EMSA showing that YY1 binds to the RGS22 promoter. The wild-type probe was incubated without (lane 1) or with (lane 2) PANC-1-YY1 cell nuclear proteins in the absence or presence of unlabeled probes (lanes 3-6). Lanes 3 and 4 contain the wild-type probe, and lanes 5 and 6 contain the mutant probe, each at 50- and 100-fold molar excess. EMSA was performed using an anti-YY1 antibody (lane 7), and the IgG antibody was used as a negative control for the YY1 antibody (lane 8). (F) Luciferase assays demonstrated the luciferase activity of PANC-1 cells transfected with YY1-overexpression or control lentiviruses. Data are presented as the mean ± SD of three independent experiments. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. EMSA, electrophoretic mobility shift assay; RGS22, Regulator of G-protein signaling 22; YY1, Yin Yang-1; OE, overexpression; NC, negative control; KD, knockdown; WT, wildtype; MT, mutant.

the clinical findings, which showed that RGS22 was overexpressed in PDAC tissues and was correlated with a better prognosis for PDAC patients. Our previous ChIP-Seq results indicated that the transcription factor YY1 could directly bind to the promoter region of RGS22, and the present results indicate that YY1 binds to a specific region of the promotor to positively regulate RGS22 expression. Thus, RGS22 may play an important role in the tumor-suppressing effect of YY1 in pancreatic cancer.

High expression of RGS22 in PDACs was shown using the IHC analysis of the 52 pairs of tissues obtained from

PDAC patients and the results of *in vitro* western blotting and RT-qPCR experiments on PDAC cells, which were consistent with data from TCGA and GTEx (20). A previous study analyzed the expression of RGS22 in several tumor types using RT-qPCR and IHC and found that RGS22 is specifically expressed in the testis and in epithelial cancers (16). This is consistent with the results of the present study, as pancreatic cancer is a classical tumor of epithelial origin.

The results of the present study showed that RGSS22 exerted a protective effect in pancreatic cancer. Data from TCGA and GTEx and the clinical data of the 52 pancreatic patients
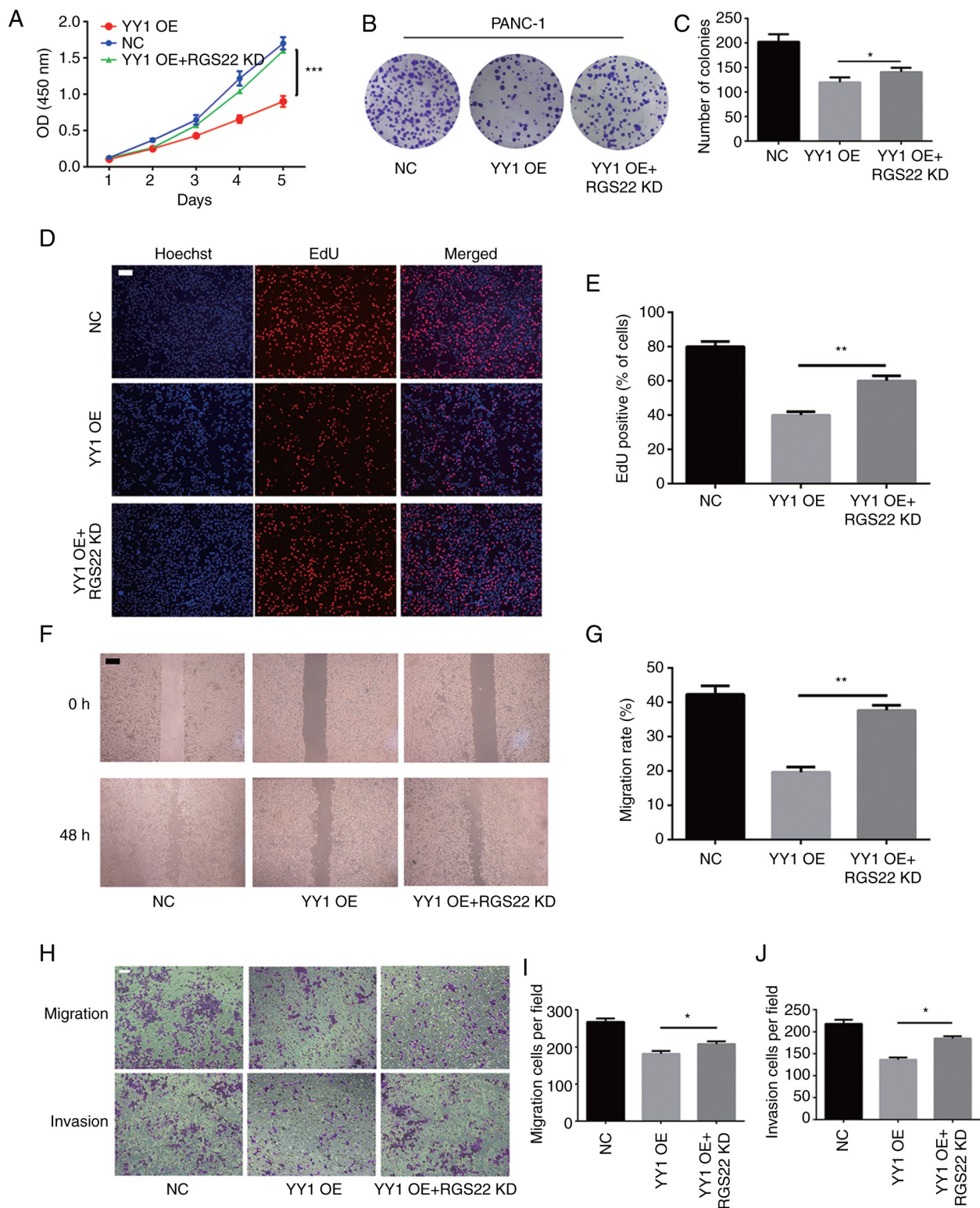
Figure 6. RGS22 as a functional target of YY1. (A) Cell Counting Kit-8, (B and C) colony formation, and (D and E) EdU assays were performed to analyze the proliferation of PANC-1-YY1 cells transfected with RGS22 siRNA. (F and G) Wound healing assays were performed to analyze the migration of PANC-1-YY1 cells transfected with RGS22 siRNA. (H-J) Cell migration and invasion assays were performed in PANC-1-YY1 cells transfected with RGS22 siRNA (magnification, x100; scale bar, 100 μm). Data are presented as the mean ± SD value of three independent experiments. *P≤0.05, **P≤0.01, ***P≤0.001. RGS22, Regulator of G-protein signaling 22; YY1, Yin Yang-1; siRNA, small interfering RNA; OD, optical density; OE, overexpression; NC, negative control; KD, knockdown.

demonstrated a correlation between high RGS22 levels and longer overall survival, as well as with a low degree of blood vessel invasion. Our *in vitro* and *in vivo* studies revealed that RGS22 suppresses the proliferation, migration, and invasion of

PDAC. RGS22 was first reported in 2008 as a testis-specific gene by Hu *et al* (17), and their study highlighted RGS22 as a cancer/testis antigen. They found that RGS22 was specifically expressed in the testis and epithelial cancers and acted as a

tumor suppressor, repressing tumor cell invasion and migration. As far as we know, only one study has investigated the role of RGS22 in pancreatic cancer and reported the suppressive effect of RGS22 on cell migration and invasion, but not proliferation (18). Therefore, the present findings make an important contribution to the literature on this topic by demonstrating the *in vitro* and *in vivo* suppressive role of RGS2 in PDAC.

RGS22 is a member of the RGS family; this means that it can interact with G proteins and negatively regulate the GPCR signaling pathway (12-14,21,22). Considering the complexity of this pathway and the limited scope of the present study, we did not investigate the pathways downstream of RGS22 in pancreatic cancer. However, we investigated the involvement of the transcription factor YY1, which is an upstream regulator of RGS22. The results of EMSA and luciferase experiments showed that YY1 directly binds to the promotor region of RGS22 and positively regulates its expression. Moreover, the recovery experiments proved that RGS22 is a functional target of YY1; this means that YY1 inhibits the proliferation, invasion, and migration of pancreatic cells by targeting RGS22.

The expression pattern of RGS22 and its function in pancreatic cancer seem to be contradictory: That is, it is overexpressed in cancer cells, but it acts as a tumor suppressor. It is hypothesized that RGS22 is not a causal factor of PDAC but is part of a feedback mechanism for the prevention of cancer progression. While RGS22 cannot prevent pancreatic cancer, it may be able to delay tumor progression and inhibit tumor metastasis. Although RGS22 expression is relatively high in pancreatic cancer cells compared with normal pancreatic cells, the absolute amount of RGS22 in PDAC is still very low. As data from TCGA and GTEx databases showed (20), RGS22 gene expression is only 0.4 TPM, which is 0.005x lower than the RGS22 expression levels in the testis. These findings point to the possibility of using exogenous RGS22 to prevent tumor progression in patients with pancreatic cancer.

Although there are no studies regarding the clinical application of RGS22 to the best of our knowledge, other members of the RGS protein family have been extensively studied for their clinical use. For example, it is well known that the GPCR signaling pathway is involved in a variety of pathophysiological processes, including tumor growth. GPCRs have long served as extraordinarily successful drug targets (23). However, due to the broad spectrum of GPCR action, drugs targeting GPCRs often lack specificity and tend to interfere with normal physiological processes. In contrast, the expression and action of the RGS proteins are tissue-specific; therefore, RGS proteins are better targets than upstream GPCRs in terms of specificity. To date, expression analyses, purification techniques, structural studies, cell line development, and screening methods related to RGS proteins have been developed, and these provide a solid foundation for designing drugs that target RGS22 (23). Furthermore, the present findings of the present study lay the basis for potential future treatments on RGS22 as a target for management of PDAC.

In conclusion, the present study demonstrated that RGS22 expression was upregulated in PDAC tissues, and YY1-mediated RGS22 regulation suppressed the proliferation, migration and invasion of PDAC cells.

Acknowledgements

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Author's contributions

SC, WG, and LM drafted the article. KJ and JZ critically revised the article for important intellectual content. KJ and JZ conceived and designed the study. SC, WG, LM, QC, YM, KJ and JZ contributed to acquisition of data, analysis, and interpretation of data. WG and LM confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University (approval no. 2009-SR-031) and all patients provided written informed consent. All animal experiments were performed in accordance with animal protocols approved by the Nanjing Medical University (approval no. 2022-SRFA-016).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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