Protein-bound Polysaccharide-K Inhibits Hedgehog Signaling Through Down-regulation of MAML3 and RBPJ Transcription Under Hypoxia, Suppressing the Malignant Phenotype in Pancreatic Cancer

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Abstract. *Hedgehog signaling is activated in pancreatic cancer and could be a therapeutic target. We previously demonstrated that recombination signal binding protein for immunoglobulin-kappa-J region (RBPJ) and mastermind-like 3 (MAML3) contribute to the hypoxia-induced up-regulation of Smoothened (SMO) transcription. We have also shown that protein-bound polysaccharide-K (PSK) could be effective for refractory pancreatic cancer that down-regulates SMO transcription under hypoxia. In this study, we evaluated whether the anticancer mechanism of PSK involves inhibiting RBPJ and MAML3 expression under hypoxia. PSK reduced SMO, MAML3 and RBPJ expression in pancreatic cancer cells under hypoxia. PSK also blocked RBPJ-induced invasiveness under hypoxia by inhibiting matrix metalloproteinase expression. Lastly, we showed that PSK attenuated RBPJ-induced proliferation both in vitro and in vivo. These results suggest that PSK suppresses Hedgehog signaling through down-regulation of MAML3 and RBPJ transcription under hypoxia, inhibiting the induction of a malignant phenotype in pancreatic cancer. Our results may lead to development of new treatments for refractory pancreatic cancer using PSK as a Hedgehog inhibitor.*

Patients with pancreatic cancer have an extremely poor prognosis and standard therapies are generally ineffective. The development of new effective therapeutic strategies for pancreatic cancer is needed. Hedgehog (Hh) signaling is activated in pancreatic cancer and involved in producing the malignant phenotype that includes increased proliferation and invasiveness (1-3). We previously demonstrated the possibility of using Hh inhibitors as anticancer therapies for pancreatic cancer (4, 5). Although various Smoothened (SMO) inhibitors have been developed, their effectiveness against pancreatic cancer in clinical trials has not been reported (6).

Increased attention has recently been given to understanding the roles of the tumor microenvironment, hypoxia in cancer development and responses to treatment. Hh signaling has been shown to be activated under hypoxic conditions *via* the up-regulation of SMO transcription (7, 8). We previously showed that blocking *SMO* transcription may be significantly more effective than inhibiting SMO protein (9). Taken together, establishing a means of suppressing the transcriptional up-regulation of *SMO* under hypoxia may be important for improving the effectiveness of Hh inhibitors and the development of new therapeutic strategies against pancreatic cancer.

Polysaccharide-K (PSK), a protein-bound polysaccharide, was developed in Japan and has been shown to be effective in combination with a variety of chemotherapeutic agents in clinical trials (10-13). PSK can suppress TGFβ1, matrix metalloproteinases (MMPs), p38 mitogen-activated protein kinase pathway activation and activated-signal transducers and activator of transcription-3 inhibiting cell invasiveness and inducing apoptosis (14-16). In addition, it was shown that PSK enhances docetaxel chemosensitivity through nuclear factor-kappa B activation in pancreatic cancer (17). Previously, we reported that PSK reduces hypoxia-induced *SMO* transcription (18). We also showed that recombination signal binding protein for immunoglobulin-kappa-J region

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(RBPJ) and mastermind-like 3 (MAML3) contribute to the hypoxia-induced up-regulation of SMO transcription (19). In this study, we investigated whether PSK contributes to the inhibition of RBPJ and MAML3 expression under hypoxia to develop a new therapeutic strategy for pancreatic cancer.

Materials and Methods

Cell culture and reagents. Two human pancreatic ductal adenocarcinoma cells (PDAC) lines (SUIT-2 and Panc-1) were maintained in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal calf serum (FCS; Life Technologies Grand Island, NY, USA), USA and antibiotics (100 units/ml of penicillin and 100 μg/ml of streptomycin). For hypoxic conditions, cells were cultured in 1% O_2, 5% CO_2, and 94% N_2 using a multigas incubator (Sanyo, Tokyo, Japan). PSK (100 μg/ml) was added to the culture and then, cell numbers were counted by light microscopy at the indicated days. PSK was kindly provided from KUREHA Co. Ltd.

Matrigel invasion assay. The invasiveness of pancreatic cancer cells was assessed based on the invasion of cells through Matrigel-coated transwell inserts, as described previously (7). In brief, the upper surface of a filter (pore size, 8.0 μm; BD Biosciences, Heidelberg, Germany) was coated with basement membrane Matrigel (BD Biosciences). Cells were suspended in RPMI-1640 with 10% FBS. Then 0.8×10^5 cells were added to the upper chamber and incubated for 16 h under hypoxia. After incubation, the filters were fixed and stained with Diff-Quick reagent (International Reagents, Kobe, Japan). All cells that had migrated from the upper to the lower side of the filter were counted under a light microscope (BX50; Olympus, Tokyo, Japan). Tumor cell invasiveness testing was carried out in triplicate wells.

Plasmid. Plasmids pFN21A HaloTag CMV Flexi-RBPJ vector and pFN21AB5901 control empty vector were purchased from Promega (Madison, WI, USA). Cells (0.2×10^6 cells/well) seeded in 6-well plates were transfected with 2.5 μg plasmids under normoxia using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. One day after transfection under normoxia, transfected PDAC were cultured with or without 100 μg/ml PSK for 2 days under hypoxia and mRNA and protein levels were estimated. In the invasion assay, after transfection, PDAC cells were cultured with or without 100 μg/ml PSK for 16 h under hypoxia. In the proliferation assay, after transfection, PDAC were cultured with or without 100 μg/ml PSK under hypoxia, and cell number was counted at intervals.

Real time polymerase chain reaction (PCR). Total RNA was extracted from wild-type PDAC with or without 100 μg/ml PSK, and control plasmid- or RBPJ plasmid-transfected PDAC with or without 100 μg/ml PSK using the High Pure RNA Isolation Kit (Roche, Mannheim, Germany), and quantified by spectrophotometry (Ultrospec 2100 Pro; Amersham Pharmacia Biotech, Cambridge, UK). For real-time RT-PCR, 1 μg of RNA was treated with DNase and reverse transcribed to cDNA with the Quantitect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Reactions were run with iQ™ SYBR Green Supermix (Bio-Rad), on a DNA Engine Option 2 System (MJ Research, Waltham, MA, USA). All primer sets amplified fragments less than 200 bp long. The primer sequences used were as follows: *SMO*, forward: CAGGTGGATGGGGACTCTGTGAGT, reverse: GAGTCATGACTCCTCGGATGAGG; *MAML3*, forward: 5'-AAGCCCAGGGACCGAGGCAA-3', reverse: 5'-GCAGCCTT GGAGGGGCTTGG-3'; *RBPJ*, forward: 5'-CGCATTATTGG ATGCAGATG-3', reverse: 5'-CAGGAAGCGCCATCATTTAT-3'; matrix metalloproteinase (*MMP9*), forward: 5'-TGGGCTACGTG ACCTATGACAT-3', reverse: 5'-GCCCAGCCCACCTCCACTC CTC-3'; *MMP2*, forward: 5'-TGATCTTGACCAGAATACCATC GA-3', reverse: 5'-GGCTTGCGAGGGAAGAAGTT-3'; E-cadherin (*CDH1*), forward: 5'-GAACAGCACGTACACAGCCCT-3', reverse: 5'-GCAGAAGTGTCCCTGTTCCAG-3', and β-actin (*ACTB*) forward: 5'-TTGCCGACAGGATGCAGAAGGA-3', and reverse: 5'-AGGTGGACAGCGAGGCCAGGAT-3'. The amount of each target gene in a given sample was normalized to the level of β-actin.

Immunoblotting. Whole-cell extraction was performed with M-PER Reagents (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Protein concentration was determined with Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA), and protein samples (50 μg) were separated by electrophoresis on an sodium dodecyl sulfate-polyacrylamide gel and transferred to Protran nitrocellulose membranes (Whatman GmbH, Dassel, Germany). Blots were then incubated with anti-SMO (1:200, sc-13943; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-MAML3 (1:100, sc-82220; Santa Cruz Biotechnology), anti-RBPJ (1:100, sc-8213; Santa Cruz Biotechnology), anti-MMP2 (1:100, sc-10736; Santa Cruz Biotechnology), anti-MMP9 (1:100, sc-6840; Santa Cruz Biotechnology) and anti-α-tubulin (1:1000; Sigma Aldrich Co., St. Louis, MO, USA) overnight at 4˚C. Blots were then incubated with the appropriate horseradish peroxidase-linked secondary antibody (Amersham Biosciences, Piscataway, NJ, USA) at room temperature for 1 hour. Immunocomplexes were detected with ECL plus Western Blotting Detection System (Amersham Biosciences) and visualized with a Molecular Imager FX (Bio-Rad). We used α-tubulin as a protein loading control.

In vivo xenograft tumor model. SUIT-2 cells (0.5×10^6 cells) transfected with *RBPJ* plasmid or control empty plasmid in 50 μl RPMI medium were subcutaneously injected into three BALB/c female nude mice (5 weeks old) in each group. All animals were obtained from The Charles River Laboratory (Wilmington, MA, USA) and maintained in standard conditions according to institutional guidelines. These animal experiments were approved by the Ethics Committee of Kyushu University (A27-004-0). Primary tumor size was measured every 4-5 days with calipers; approximate tumor weights were determined using the formula $0.5ab^2$, where a is the larger and b is the smaller of the two perpendicular diameters.

PSK dosage was determined by reference to the previous study (10). PSK (6 mg/mouse) was intraperitoneally injected twice a week. No toxic side-effect was observed in any mice by inspection.

Statistical analysis. The data are presented as the means±standard deviation (SD). Student's *t*-tests were used to compare continuous variables between two groups throughout this study using Microsoft Excel software (Microsoft Corp., Redmond, WA, USA). *p*-Values of <0.05 were considered as statistically significant.

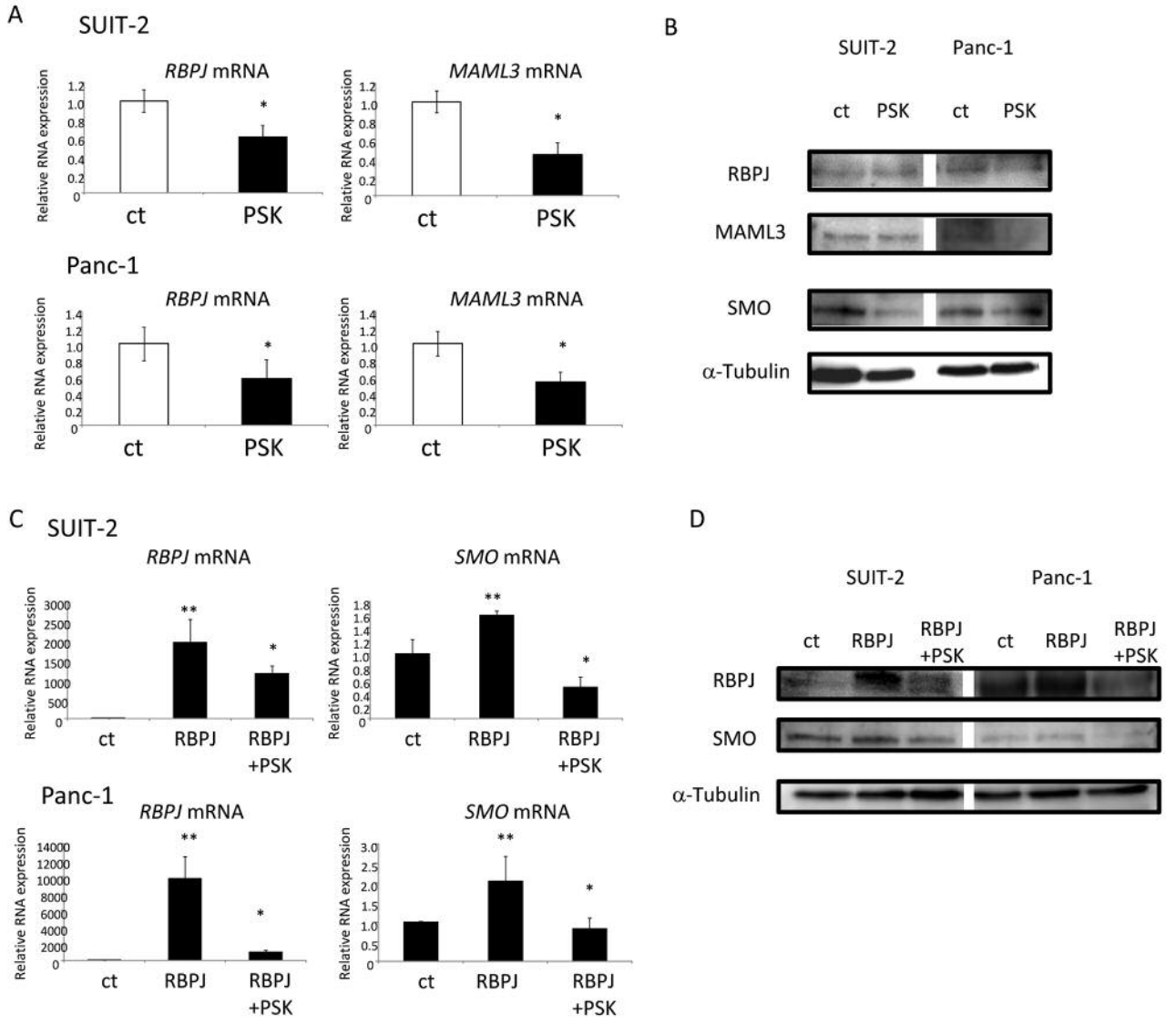

Figure 1. *Protein-bound polysaccharide-K (PSK) reduces mastermind-like 3 (MAML3) and recombination signal binding protein for immunoglobulin-kappa-J region (RBPJ) expression in pancreatic ductal adenocarcinoma cells (PDAC) under hypoxia. A: RBPJ and MAML3 mRNA levels in PDAC under hypoxia were measured by real-time polymerase chain reaction (PCR) following 2-day treatment with or without (ct) PSK. *p<0.05. B: RBPJ, MAML3 and Smoothened (SMO) protein levels in PDAC under hypoxia following 2-day treatment with PSK were investigated by western blotting; α-tubulin was used as a loading control. The mRNA (C) and protein (D) levels of RBPJ and SMO in RBPJ-transfected PDAC with and without (ct) PSK treatment were measured by real-time PCR and western blotting, respectively. In these experiments, RBPJ-expression or control plasmids were transfected under normoxia for 1 day. Then transfected PDAC were cultured with or without PSK for 2 days under hypoxia. Bars show the mean, and error bars show the standard deviation. *p<0.05 Compared with the RBPJ plus PSK group; **p<0.05 compared with the RBPJ group.*

Results

PSK reduces MAML3 and RBPJ expression in PDAC cells under hypoxia. *RBPJ, MAML3* and *SMO* expression was measured by real-time PCR and western blotting in PSK-treated cells under hypoxia, and the data showed that in all three cases, expression significantly decreased in PSK-treated cells compared with control cells also grown in low oxygen (Figure 1A and B). Next, an *RBPJ* expression plasmid was transfected into the cells, and *RBPJ* and *SMO* mRNA and protein levels were found increased compared to control-transfected cells. PSK suppressed *RBPJ* and *SMO* mRNA and protein expression in RBPJ-transfected cells (Figure 1C and D). These results suggest that PSK reduces *MAML3* and *RBPJ* expression in PDAC under hypoxic conditions.

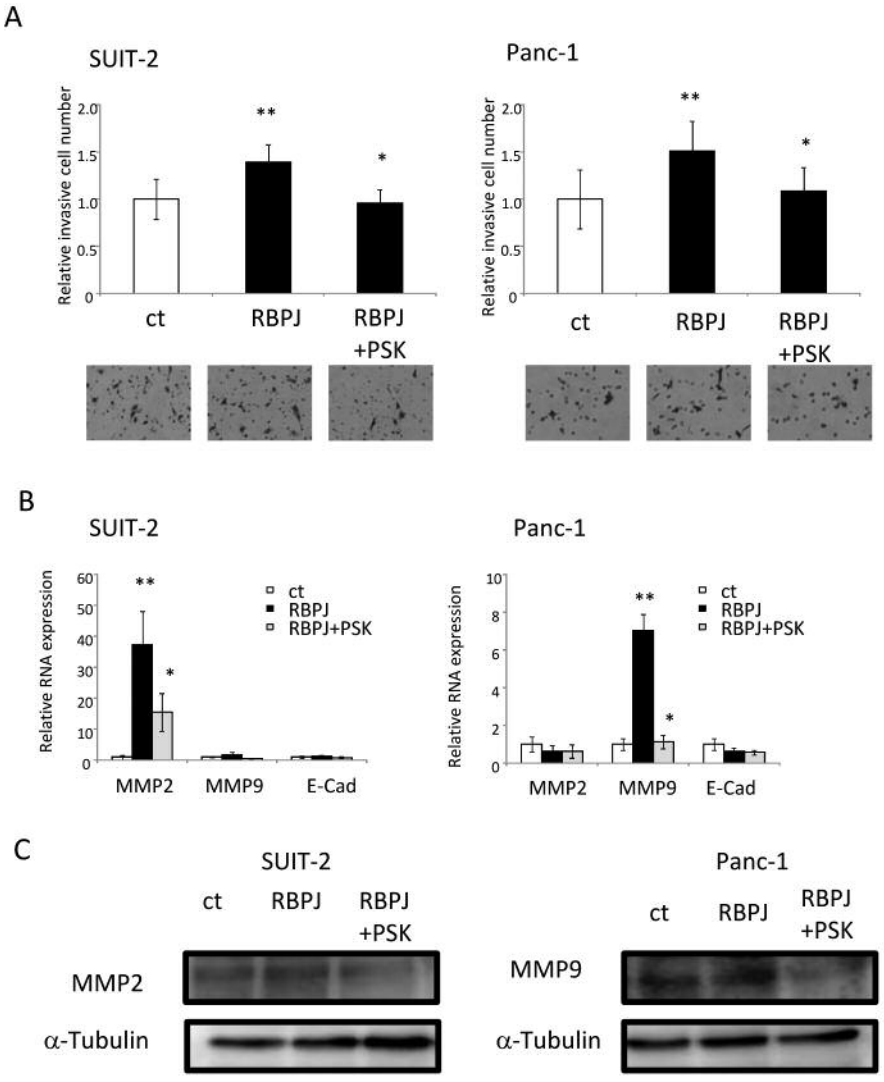

Figure 2. *Protein-bound polysaccharide-K (PSK) blocks recombination signal binding protein for immunoglobulin-kappa-J region (RBPJ)-induced invasiveness under hypoxia through matrix metalloproteinase (MMP) inhibition. A: Following transfection with RBPJ or control plasmids for 1 day under normoxia, invasiveness of transfected pancreatic ductal adenocarcinoma cells (PDAC) with and without (ct) PSK treatment was analyzed in the Matrigel invasion assay under hypoxia. The graph shows the number of invading cells, and representative images are shown; original magnification ×100. B: Following transfection with RBPJ or control plasmids for 1 day under normoxia, MMP2, MMP9 and E-cadherin (E-Cad) mRNA expression in the transfected PDAC with and without (ct) 2-day PSK treatment under hypoxia were measured by real-time polymerase chain reaction. C: MMP2 and MMP9 protein expression was also analyzed by western blotting. This experiment was a continuation of the analysis presented in Figure 1D. The α-tubulin loading control blot is therefore repeated from Figure 1D. Bars show the mean, and error bars show the standard deviation. *p<0.05 Compared to RBPJ plus PSK group; **p<0.05 compared with the RBPJ group.*

PSK blocks RBPJ-induced invasiveness under hypoxia through MMP inhibition. We have previously shown that PSK blocks invasiveness by inhibiting *SMO* transcription (10) and that RBPJ contributes to SMO transcription under hypoxia (19). Here we analyzed whether RBPJ regulation contributes to the decreased invasiveness of PSK-treated cells. Invasiveness of *RBPJ*-transfected cells was significantly higher than in control cells, and PSK treatment blocked *RBPJ*-induced invasiveness (Figure 2A). We have also shown that MMPs contribute to Hh signaling-induced invasiveness in PDAC (4, 7). Therefore, we next investigated whether MMPs are involved in *RBPJ*-induced invasiveness. Interestingly, MMP2 was shown to contribute to the invasiveness of SUIT-2 cells, and MMP9 is involved in the invasiveness of Panc-1 cells (Figure 2B and C). On the other hand, there were no significant changes in E-cadherin expression in any of the treatment groups, suggesting that endothelial–mesenchymal transition (EMT) might not contribute to that invasiveness (Figure 2B and C).

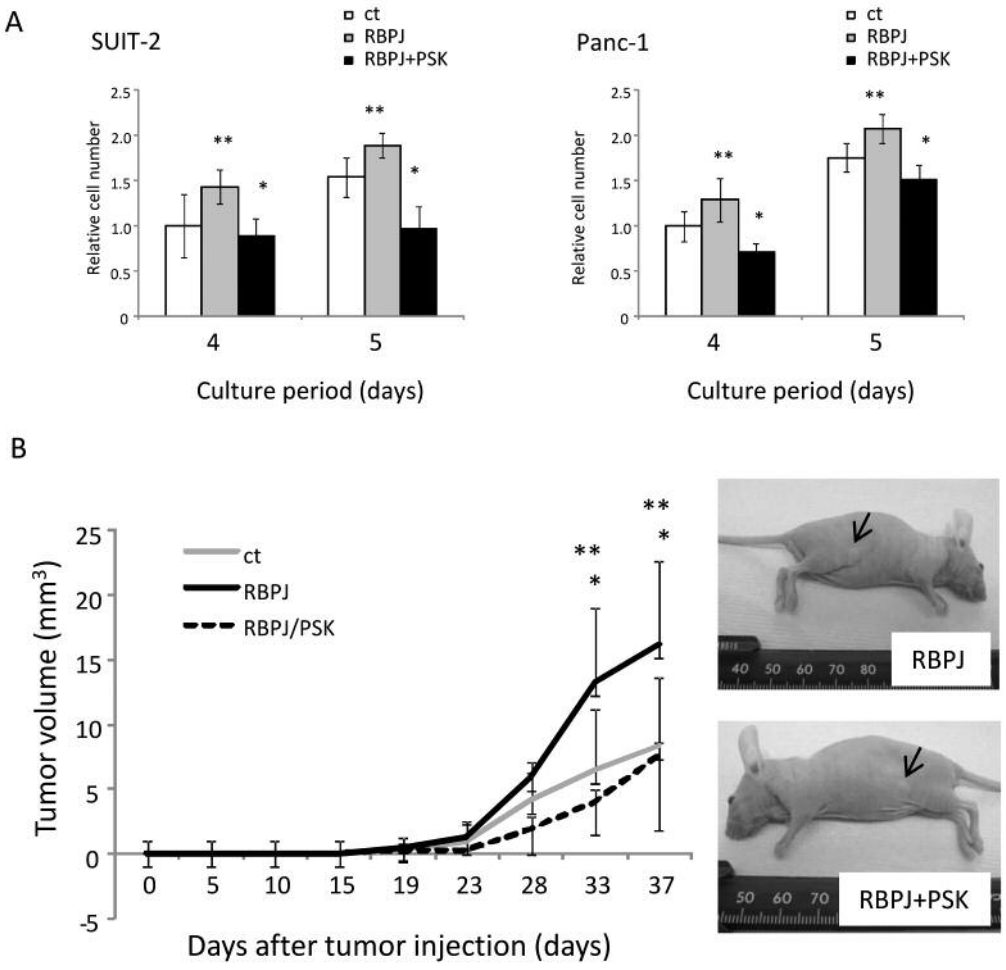

Figure 3. *Protein-bound polysaccharide-K (PSK) attenuates recombination signal binding protein for immunoglobulin-kappa-J region (RBPJ)-induced proliferation both in vitro and in vivo. A: Following transfection with RBPJ or control plasmids for 1 day under normoxia, proliferation under hypoxia in transfected pancreatic ductal adenocarcinoma cells with or without (ct) PSK treatment was measured. B: Following transfection with RBPJ or control plasmids, 0.5×10^6 SUIT-2 cells in 50 μl RPMI medium were subcutaneously injected into three 5-week-old BALB/c female nude mice per group. PSK (6 mg/mouse) was intraperitoneally injected twice a week. Primary tumor size was measured every 4-5 days. Bars show the mean and error bars show standard deviation. *p<0.05 Compared to RBPJ plus PSK group; **p<0.05 compared with the RBPJ group.*

PSK attenuates RBPJ-induced proliferation both in vitro and in vivo. Increased proliferation and invasion are two of the malignant phenotypes of pancreatic cancer. We have previously demonstrated that PSK reduces the proliferative rate of pancreatic cancer cells by inhibiting SMO transcription (10). Here, we analyzed whether RBPJ regulation contributes to the decreased proliferation of PSK-treated cells. The proliferation rate of *RBPJ*-transfected cells was significantly higher than that of control cells, and PSK blocked *RBPJ*-induced proliferation *in vitro* (Figure 3A).

Next, we confirmed these *in vitro* results using a mouse model. Tumor volumes from mice subcutaneously injected with *RBPJ*-transfected SUIT-2 cells were significantly higher than those from mice injected with SUIT-2 cells transfected with the control empty plasmid (Figure 3B). As expected, PSK attenuated *RBPJ*-induced proliferation *in vivo*, approximately to the same level as control cells (Figure 3B). These results suggest that PSK blocks *RBPJ*-induced proliferation both *in vitro* and *in vivo*.

Discussion

In this study, exogenous *RBPJ* overexpression using an *RBPJ* expression plasmid did not induce major phenotypic differences in PDAC. This may be because RBPJ is sufficiently expressed in PDAC to induce biological effects. However, as shown in a previous study, suppressing *RBPJ* dramatically reduced proliferation and invasion in PDAC

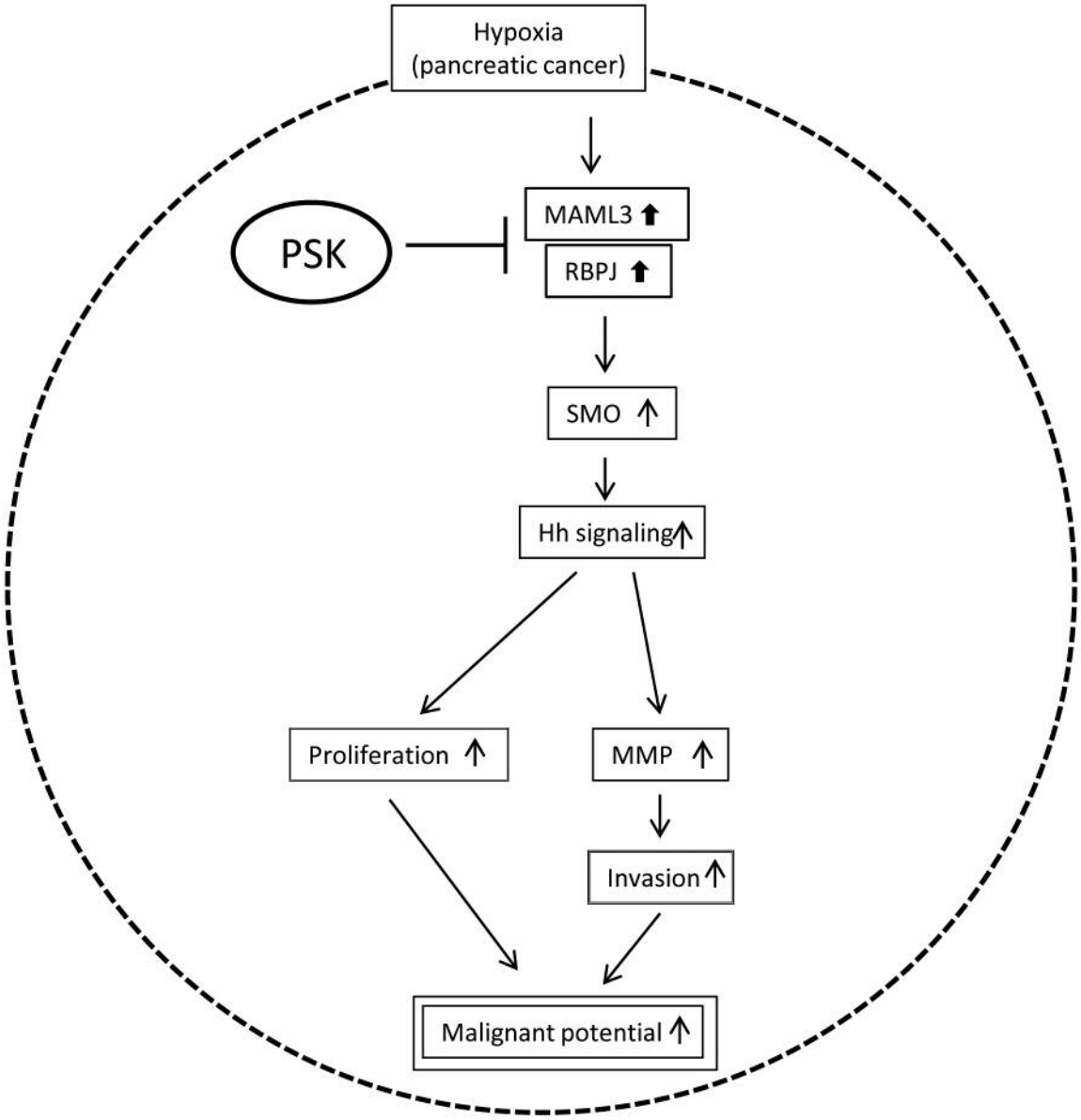

Figure 4. *Schematic of the interaction between Protein-bound polysaccharide-K (PSK) and hedgehog (Hh) signaling in pancreatic cancer. Smoothened (SMO) mRNA transcription may be increased by the up-regulation of mastermind-like 3 (MAML3) and recombination signal binding protein for immunoglobulin-kappa-J region (RBPJ) under hypoxia (19). PSK may reduce MAML3 and RBPJ expression under hypoxia, inhibiting SMO transcription and suppressing the malignant phenotype of pancreatic ductal adenocarcinoma cells. This may represent the underlying mechanism of the antitumor activity of PSK against pancreatic cancer.*

(19). We therefore conclude that RBPJ expression significantly influences malignant phenotypes in PDAC.

Interestingly, RBPJ and MAML3 are also known activators of NOTCH signaling, which like Hh signaling, is involved in embryonic morphogenesis. It has been shown that hypoxia activates NOTCH signaling through hypoxia-inducible factor 1α (HIF1α) activation (20-21). Qiang *et al.* revealed that HIF1α mediates hypoxia-mediated maintenance of glioblastoma stem cells through NOTCH activation (22). However, our preliminary results suggested that HIF1α was not involved in inducing the expression of *SMO*, glioma-associated oncogene1 (*GLI1*), *RBPJ* and *MAML3* (7, 19). Further research into the relationships among *RBPJ, MAML3* and *HIF1α* is required to fully understand the mechanism underlying the hypoxia-induced activation of NOTCH signaling. However, because PSK can inhibit the expression

of *SMO, RBPJ* and *MAML3*, it may have value as a pan-inhibitor of the reactivated morphogenic signaling pathways in malignant pancreatic tumors.

As previously described, PSK suppresses both *HIF1α* and *SMO* expression (10). PSK is a polysaccharide, not a purified single material, and is difficult to separate into a single species. It is, therefore, unclear which component molecule in PSK contributes to the repression of *MAML3* and *RBPJ* expression. In a previous study, PSK was divided into two fractions: ≤10,000 normal molecular weight limit (NMWL, low components) and ≥10,000 NMWL (high components). Both components were found to inhibit Hh signaling; the high component fraction also reduced HIF1α expression (18). The molecular weight of RBPJ is 56 kDa and the molecular weight of MAML3 is 150-170 kDa. Consistent with a previous study of ours (18), both RBPJ and MAML3 may inhibit Hh and HIF1α signaling.

MMPs and EMT are thought to be important factors in cancer invasion and metastasis. The expression of MMPs correlated with *RBPJ*-induced invasiveness in this study. Interestingly, MMP2 expression regulates invasiveness in ASPC-1 and SUIT-2 cells (19), while MMP9 expression is paramount for an invasive phenotype in Panc-1 cells (Figure 2B and C). It is likely that different MMPs regulate invasiveness in different cell types. Some studies have shown that Hh and NOTCH signaling induces an invasive phenotype through EMT in PDAC (8, 23). However, the effects of EMT are not likely to have contributed to the *RBPJ*-induced invasiveness and subsequent PSK-induced inhibition of invasiveness in this study as assessed by E-cadherin expression (Figure 2B).

Figure 4 shows a schematic conclusion from the present study based on our previous findings. Hh signaling is activated through the up-regulation of SMO transcription under hypoxic conditions, inducing a malignant phenotype in PDAC (7). *RBPJ* and *MAML3* contribute to the up-regulation of *SMO* under hypoxia (19). In the present study, we demonstrated that PSK reduces *MAML3* and *RBPJ* expression under hypoxia, inhibiting *SMO* transcription and suppressing the malignant phenotype of PDAC cells. This mechanism underlies the antitumor effects of PSK against PDAC. Our results may lead to development of new treatment strategies for refractory pancreatic cancer using Hh inhibitors.

Acknowledgements

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Conflicts of Interest

All Authors declare no conflict of interest in regard to this study.

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