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A PAUF-neutralizing antibody targets both carcinoma and endothelial cells to impede pancreatic tumor progression and metastasis

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ABSTRACT

Pancreatic adenocarcinoma up-regulated factor (PAUF) is expressed in pancreatic ductal adenocarcinoma (PDAC) and plays an important role in tumor progression and metastasis. Here we evaluate the anti-tumor efficacy of a human monoclonal antibody against PAUF, PMAb83, to provide a therapeutic intervention to treat the disease. PMAb83 reduced tumor growth and distant metastasis in orthotopically xenografted mice of human PDAC cells. PMAb83 treatment retarded proliferation along with weakened aggressiveness traits of the carcinoma cells. AKT/β-catenin signaling played a role in the carcinoma cell proliferation and the treated xenograft tumors exhibited reduced levels of β-catenin and cyclin D1. Moreover PMAb83 abrogated the PAUF-induced angiogenic responses of endothelial cells, reducing the density of $CD31^+$ vessels in the treated tumors. In combination with gemcitabine, PMAb83 conferred enhanced survival of xenografted mice by about twofold compared to gemcitabine alone. Taken together, our findings show that PMAb83 treatment decreases the aggressiveness of carcinoma cells and suppresses tumor vascularization, which culminates in mitigated tumor growth and metastasis with improved survival in PDAC mouse models.

1. Introduction

Pancreatic cancer is one of the most aggressive malignancies with the worst mortality rate of all cancers. Annually, about 10 new cases of pancreatic cancer per 100,000 population and nearly as many deaths from the disease are expected [1]. Early diagnosis is infrequent and the majority of patients present with locally advanced or metastatic disease, which is surgically inoperable and resistant to conventional chemotherapy and radiotherapy [2]. Only about 15% of patients with the disease are eligible for curative resection at diagnosis, and 12–32% of the surgically resected patients will survive 5 years without evidence of recurrence [3–5]. For locally advanced or metastatic pancreatic cancer, treatment is palliative and chemotherapy remains the only option. Gemcitabine (a nucleoside analog) with or without erlotinib (an epidermal growth factor receptor tyrosine kinase inhibitor) has been the standard chemotherapeutic agent as first-line therapy, despite low response rates and modest survival benefit [6,7]. With the advance of our understanding about the molecular pathogenesis of the disease, many targeted therapeutic approaches in various treatment modalities have been explored for more than a decade [8–11]. However the outcomes of clinical trials with these targeting agents have been disappointing. There still remains, therefore, an urgent need for new therapeutic strategies to treat pancreatic cancer. Understanding the complex biology of pancreatic cancer

Abbreviations: PAUF, pancreatic adenocarcinoma up regulating factor; PDAC, pancreatic ductal adenocarcinoma; HUVEC, human umbilical vein endothelial cell.

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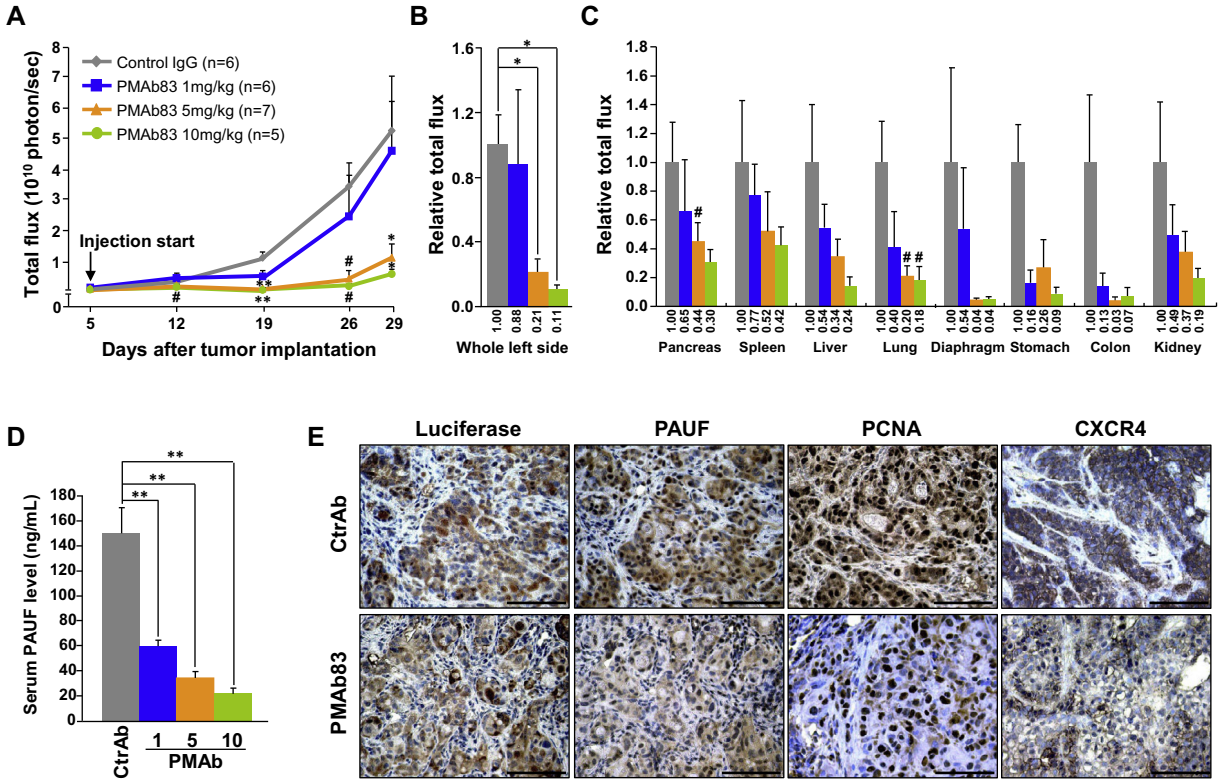

Fig. 1. PMAb83 impedes PDAC tumor progression and metastasis in vivo. CFPAC-1 cells stably expressing the firefly luciferase gene were implanted into the pancreas of mice and treated with control IgG (10 mg/kg) or PMAb83 (1, 5, or 10 mg/kg) intraperitoneally twice a week. (A) Tumor growth was presented by total flux from bioluminescence imaging in vivo. (B) At day 29 post-implantation, in vivo total flux of left side of the body was measured. (C) Mice were sacrificed to measure total flux of individual organs ex vivo. The numbers below the graph indicate relative total flux to the control IgG group. (D) Serum PAUF level of mice was measured by ELISA. (E) Primary tumors were used for immunohistochemical staining to detect luciferase, PAUF, PCNA, and CXCR4. Scale bar, 100 μm. $^{\#}P < 0.05$; $^{*}P < 0.01$; $^{**}P < 0.001$.

at the genome-wide level may lead to the identification of key factors for the development of the disease. Genomic analyses have demonstrated that many cellular signaling pathways and processes are either genetically or epigenetically altered [12–14]. Results from analyses of genomic mutations [15,16] and a mouse model [17] of the disease implicate a broad time window of opportunity for early intervention. Through genome-wide analyses we have previously identified PAUF (pancreatic adenocarcinoma up-regulated factor), which is a secreted protein highly expressed in human pancreatic ductal adenocarcinoma (PDAC) tissues when compared to expression levels in normal pancreas [18]. Moreover, PAUF positively expresses in majority of patients with pancreatic cancer, suggesting its roles in pancreatic cancer development [19]. Functioning as an autocrine factor, varied PAUF levels result in altered migration, invasion, proliferation, and adhesion of pancreatic carcinoma cells [19–21] and correlate with the metastasis of the cells to distant organs in orthotopically xenografted mouse models [19]. In paracrine manners, PAUF creates a microenvironment favorable for tumor progression and metastasis. PAUF activates monocytes to produce pro-tumorigenic cytokines, and consequently facilitates tumor growth and escape from innate immune surveillance [22]. PAUF contributes toward angiogenesis by stimulating activation of endothelial cells and increased vascular permeability [23]. In this report, we show that a fully human anti-PAUF monoclonal antibody inhibits pancreatic tumor progression and metastasis by affecting both carcinoma and endothelial cells. Our experimental results provide a rationale for the development of a novel and effective molecularly targeted therapeutic intervention for pancreatic cancer treatment.

2. Materials and methods

2.1. Cell culture and reagents

Human PDAC cell lines (CFPAC-1, BxPC-3) were obtained from the American Type Culture Collection (Manassas, VA, USA). Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Basel, Switzerland). All the reagents used in this study are described in the Supplementary materials and methods.

2.2. Generation of PAUF-specific monoclonal antibody

PMAb83, a human monoclonal antibody against PAUF, was from a human phage-antibody library. Detailed description of this process can be found in the Supplementary materials and methods.

2.3. In vivo animal studies

All animal studies were performed in compliance with the policy of the KRIBB Animal Care and Use Committee. Five-week-old BALB/c nude female mice were used. Detailed description of the methods can be found in the Supplementary materials and methods.

2.4. In vitro cell-based studies

Effects of PMAb83 on pancreatic carcinoma cells were examined using in vitro cell-based assays including proliferation,

migration, invasion, adhesion, and anoikis resistance assays. In vitro angiogenesis assays were performed for HUVECs. Western blot analyses, immunohistochemical and immunocytochemical stainings were performed according to standard protocols. Detailed description of the methods can be found in the Supplementary materials and methods.

2.5. Statistical analyses

Data were presented as mean ± SD for in vivo data or ± SE for in vitro data, and the Student *t* test was used to determine statistical significance. $P < 0.05$ was considered statistically significant.

3. Results

3.1. PMAb83 impedes pancreatic tumor cell progression and metastasis in vivo

The human monoclonal antibody PMAb83 was generated from human scFv phage library based on its reactivity to PAUF. PMAb83 was shown to bind specifically to PAUF with a high affinity ($Kd = 5 \times 10^{-9}$ M) (Data not shown). The therapeutic potential of PMAb83 was examined in luminescence-based orthotopic pancreatic tumor mouse models. PDAC cells CFPAC-1 or BxPC-3, endogenously expressing PAUF at high levels (Supplementary Table 1), were implanted into pancreas of nude mice. PDAC tumor-bearing mice injected with PMAb83 showed markedly reduced signal intensities in dose dependent manners compared with control immunoglobulin G (IgG) mice (Fig. 1A, B and Supplementary Fig. 1A, B). Ex vivo imaging of individual organs excised from the mice revealed that the ability of PDAC cells to locally invade and metastasize to distant organs was drastically interrupted by PMAb83 treatment (Fig. 1C and Supplementary Fig. 1C). In addition, PMAb83 also attenuated the tumor growth and metastasis in mice with advanced tumors (Supplementary Fig. 2). The treatment reduced both serum PAUF level judged by ELISA (Fig. 1D and Supplementary Fig. 1D) and PAUF staining in xenograft tumors judged by immunohistochemical analyses (Fig. 1E), indicating effective targeting of PMAb83 against PAUF. Reduced proliferating cell nuclear antigen (PCNA) level in the treated tumors suggested anti-proliferative effect of PMAb83 (Fig. 1E). Moreover C-X-C chemokine receptor 4 (CXCR4) expression, induced by PAUF and proposed to be associated with enhanced metastasis of carcinoma cells [20], was also reduced by PMAb83. These data indicate that PMAb83 can effectively impair in vivo tumor progression and metastasis by specifically targeting PAUF.

3.2. PMAb83 attenuates the tumorigenic traits of pancreatic carcinoma cells

Based on our previous observations that PAUF strengthens the cancer-associated traits of PDAC cells [19,20], we next explored the effects of PMAb83 at the cellular and molecular levels. PMAb83 inhibited the proliferation of CFPAC-1 and BxPC-3 cells in a

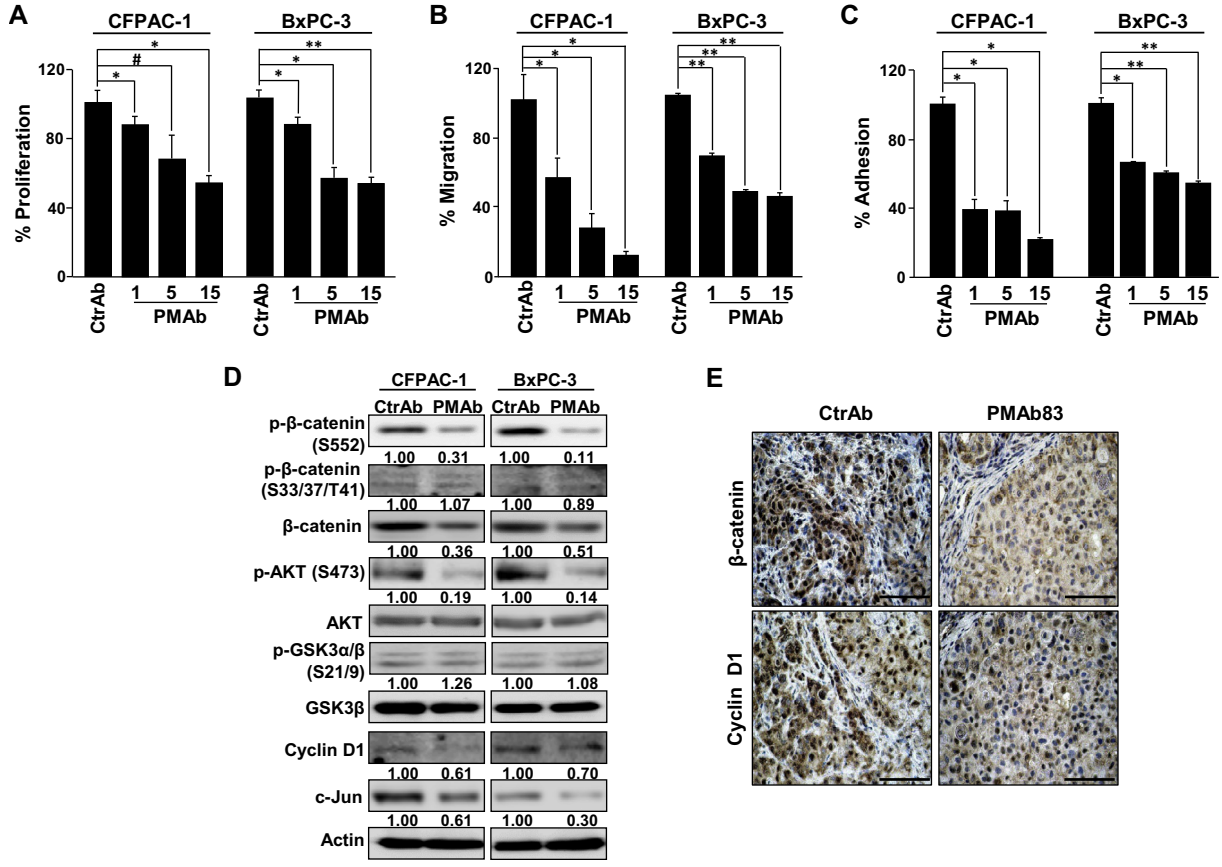

Fig. 2. PMAb83 attenuates the aggressiveness of PDAC cells. (A–C) CFPAC-1 or BxPC-3 cells were incubated in the presence of PMAb83 (1, 5, or 15 μg/mL) or control IgG (15 μg/mL). (A) Cell proliferation was determined by Alamar blue assay. (B) Cell migration was assessed using Transwell chambers. (C) Cell adhesion was determined using collagen type I-coated plates. Results were from three independent experiments with triplicate samples in each. $^{\#}P < 0.05$; $^{*}P < 0.01$; $^{**}P < 0.001$. (D) Serum-starved CFPAC-1 or BxPC-3 cells were incubated with PMAb83 (15 μg/mL) or control IgG (15 μg/mL) for 12 h. Cell lysates were prepared and used for Western analysis with the indicated antibodies. Actin was used as a loading control. Signals were quantified by ImageJ and presented as the ratios of the band intensities relative to their respective controls. (E) Primary tumors of Fig. 1 were used for immunohistochemical staining to detect β-catenin and cyclin D1. Scale bar, 100 μm.

dose-dependent manner (Fig. 2A). The motility (Fig. 2B), invasiveness (Supplementary Fig. 3A), and adhesiveness (Fig. 2C) of the carcinoma cells were also significantly inhibited by PMAb83 treatment. Similarly the treatment decreased resistance of the carcinoma cells to anoikis-mediated cell death (Supplementary Fig. 3B). PAUF-induced cell proliferation is attributable in part to increased β-catenin level [21]. It has been shown that activated AKT can phosphorylate β-catenin either directly at the C-terminus, leading to the stabilization and nuclear accumulation, and/or indirectly at the N-terminus via glycogen synthase kinase 3 beta (GSK-3β), which triggers proteasomal degradation [24,25]. In PMAb83-treated PDAC cells, concomitant with reduced AKT activation, the phosphorylation level of β-catenin at C-terminal Ser552 was greatly diminished, whereas those of β-catenin at N-terminal Ser33/37/Thr41 and GSK-3β were unaffected (Fig. 2D). Accordingly, immunohistochemical staining revealed decreased the levels of β-catenin in nuclei of the treated xenograft tumors (Fig. 2E). Reduced expression levels of the downstream target molecules of β-catenin, cyclin D1 and c-Jun, was also demonstrated in PDAC cells and the treated tumors (Fig. 2D and E). Our results suggest that β-catenin in PDAC cells is stabilized to localize in nucleus by its C-terminal phosphorylation, which can be reversed by PMAb83.

3.3. PMAb83 reduces angiogenesis by abrogating endothelial cell activation

Since PAUF has a paracrine function to induce angiogenesis [23], we examined PMAb83's effects on endothelial cells. PMAb83 significantly reduced the proliferation, migration and capillary-like tube formation of PAUF-activated HUVECs (Fig. 3A–C). PMAb83 also blocked PAUF-stimulated microvessel sprouting in an ex vivo mouse aortic ring assay, and decreased PAUF-induced blood vessel formation in vivo, as measured by Matrigel plug assay (Supplementary Fig. 4). When we assessed the effects of PMAb83 on vascular permeability, PMAb83 decreased fluorescein isothiocyanate (FITC)-dextran permeability (Fig. 3D) and alleviated VE-cadherin loss at cell–cell junctions (Fig. 3E) in HUVECs. Moreover, immunohistochemical analysis of the xenograft tumors demonstrated substantially reduced blood vessel density in the PMAb83-treated tumors (Fig. 3F). Taken together, these results suggest that the angiogenesis and vascular permeability of PDAC tumors can be effectively limited by targeting PAUF.

3.4. PMAb83, in combination with gemcitabine, improves survival

To assess the therapeutic potential of PMAb83 for the treatment of PDAC, we next examined the anti-tumor effects of PMAb83 in combination with gemcitabine. In CFPAC-1 orthotopic xenograft mice, tumors treated with PMAb83 (5 mg/kg) plus gemcitabine (20 mg/kg) grew more slowly than those treated with PMAb83 or gemcitabine alone (Fig. 4A). At 29 days post-implantation, PMAb83- and gemcitabine-treated mice showed reduced tumor growth by 70% and 66%, respectively, compared to control IgG-treated mice (Fig. 4B). Tumor growth of mice treated with PMAb83 plus gemcitabine was inhibited even further compared to those treated with PMAb83 or gemcitabine alone (Fig. 4B). Moreover ex vivo analyses of the xenograft mice demonstrated that the combined treatment reduced the incidence of distant tumor metastases, especially to the liver and lung (Fig. 4C). Importantly Kaplan–Meier survival analysis revealed a substantial survival benefit for combination-treated mice (Fig. 4D). The median survival of control IgG-treated mice was 33 days. Survival was prolonged to 41 days in the gemcitabine group, 59 days in the PMAb83 group, and 78 days in the combination group. Thus, in our experimental

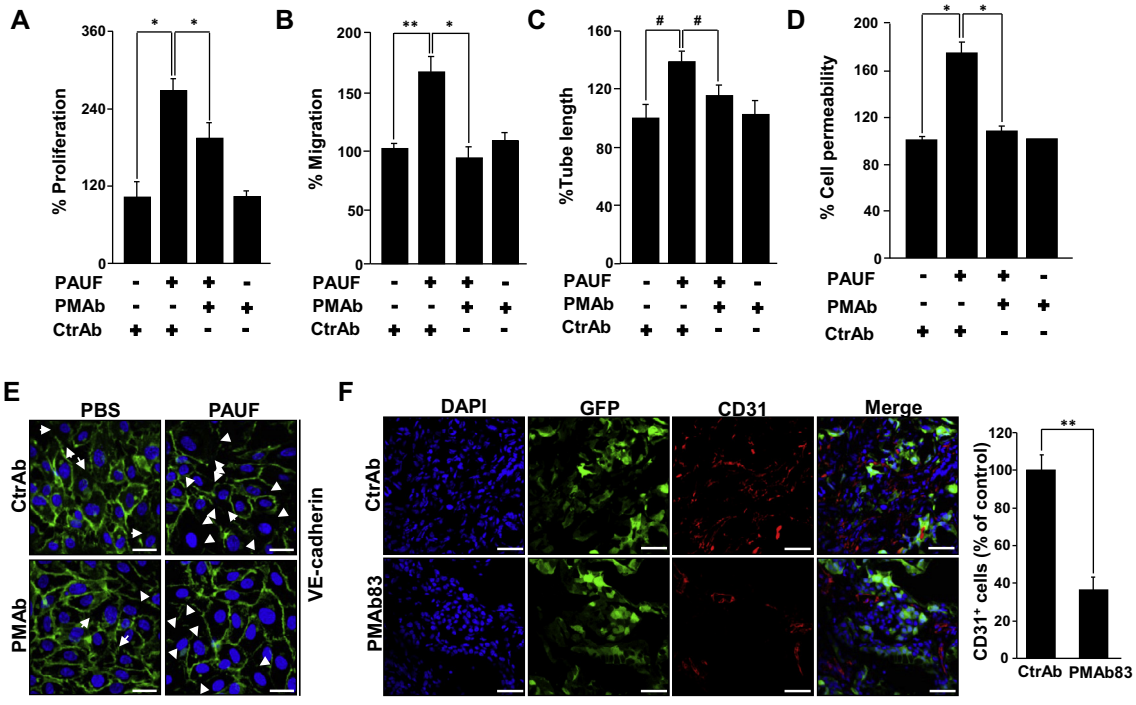

Fig. 3. PMAb83 reduces angiogenesis by abrogating endothelial cell activation. (A–D) HUVECs were incubated in the presence of control IgG (15 μg/mL) or PMAb83 (15 μg/mL) with or without PAUF (0.5 μg/mL). (A) Cell proliferation was determined by Alamar blue assay. (B) Cell migration was assessed using gelatin-coated Transwell chambers. (C) Capillary-like tube formation was determined using Matrigel-coated plates. (D) In vitro FITC-dextran permeability assay was performed in gelatin-coated chambers. Results were from three independent experiments with triplicate samples in each. $^{\#}P < 0.05$; $^{*}P < 0.01$; $^{**}P < 0.001$. (E) Immunofluorescence analysis of VE-cadherin. HUVECs were grown to confluence on gelatin-coated plates and treated with control IgG (15 μg/mL) or PMAb83 (15 μg/mL) with or without PAUF (0.5 μg/mL). Arrows indicate disruption of VE-cadherin. Scale bar, 50 μm. (F) Primary tumors of Fig. 1 were used for immunofluorescence staining of CD31. Quantitation results were presented on Right. Data are presented as relative percentage of the experimental group compared to control group. Scale bar, 50 μm. $^{*}P < 0.01$; $^{**}P < 0.001$.

mouse models, the combination of PMAb83 with gemcitabine had greater PDAC therapeutic activity than gemcitabine alone.

4. Discussion

In this study we provide lines of evidence that PMAb83, a PAUF-targeting monoclonal antibody, effectively inhibits pancreatic tumor progression and metastasis. First, PMAb83 retards tumor growth and represses the metastasis of carcinoma cells to distant organs in orthotopically xenografted mouse models. Second, PMAb83 attenuates the aggressiveness and metastatic traits of carcinoma cells. Third, PMAb83 represses tumor neo-angiogenesis by inhibiting the functional activation of endothelial cells and vascular permeability. Finally, combination treatment of PMAb83 with gemcitabine more efficiently hinders tumor progression and extends survival.

Consistent with our previous observation that PAUF promotes proliferation by increasing β-catenin levels [21], we herein show that PMAb83 significantly reduces β-catenin levels in pancreatic carcinoma cells and orthotopic tumor specimens. Activated Wnt/β-catenin signaling facilitates tumorigenesis in several human cancer types including gastrointestinal tumors [26]. While most colorectal tumors have mutations in adenomatous polyposis coli (APC) or β-catenin, about 65% of PDAC shows increased β-catenin accumulation in the nucleus and/or cytoplasm with rare mutations in key factors of Wnt/β-catenin signaling [27], implicating context dependency of the signaling. Notably, increased β-catenin levels have been reported in a mouse model of PDAC, and inhibition of Wnt signaling was found to reduce proliferation and increase apoptosis of carcinoma cells [28]. Global analyses of genetic alterations have defined a core set of 12 cellular signaling pathways and processes of pancreatic tumorigenesis, including the Wnt pathway [12]. Data in this study show that the phosphorylation level at the N-terminus of β-catenin, required for proteasomal degradation, is not altered by PMAb83 treatment. Instead, our data indicate that PAUF is responsible for inducing AKT-mediated phosphorylation of β-catenin at the C-terminus, resulting in stabilization and nuclear accumulation. Both GSK-3β and AKT are involved in modulating β-catenin levels during colorectal cancer tumorigenesis [25]. Thus, though β-catenin signaling is activated in both cancer types, the way how the pathway is activated may differentiate PDAC from colorectal cancer. We propose that the reduced proliferation of PDAC cells by PMAb83 is due, at least in part, to inhibition of PAUF-mediated AKT activation and the subsequent down-regulation of active β-catenin. Along with this anti-proliferative effect, PMAb83 might also decrease the aggressiveness of PDAC cells by inhibiting the intracellular signaling pathways involved in cell motility and adhesion.

Communication between tumor and stromal cells within a given microenvironment is instrumental in promoting tumor progression and metastasis [29]. Especially PDAC is histologically characterized by dense stromal regions comprised of several different cell types including fibroblast, immune cells, endothelial cells,

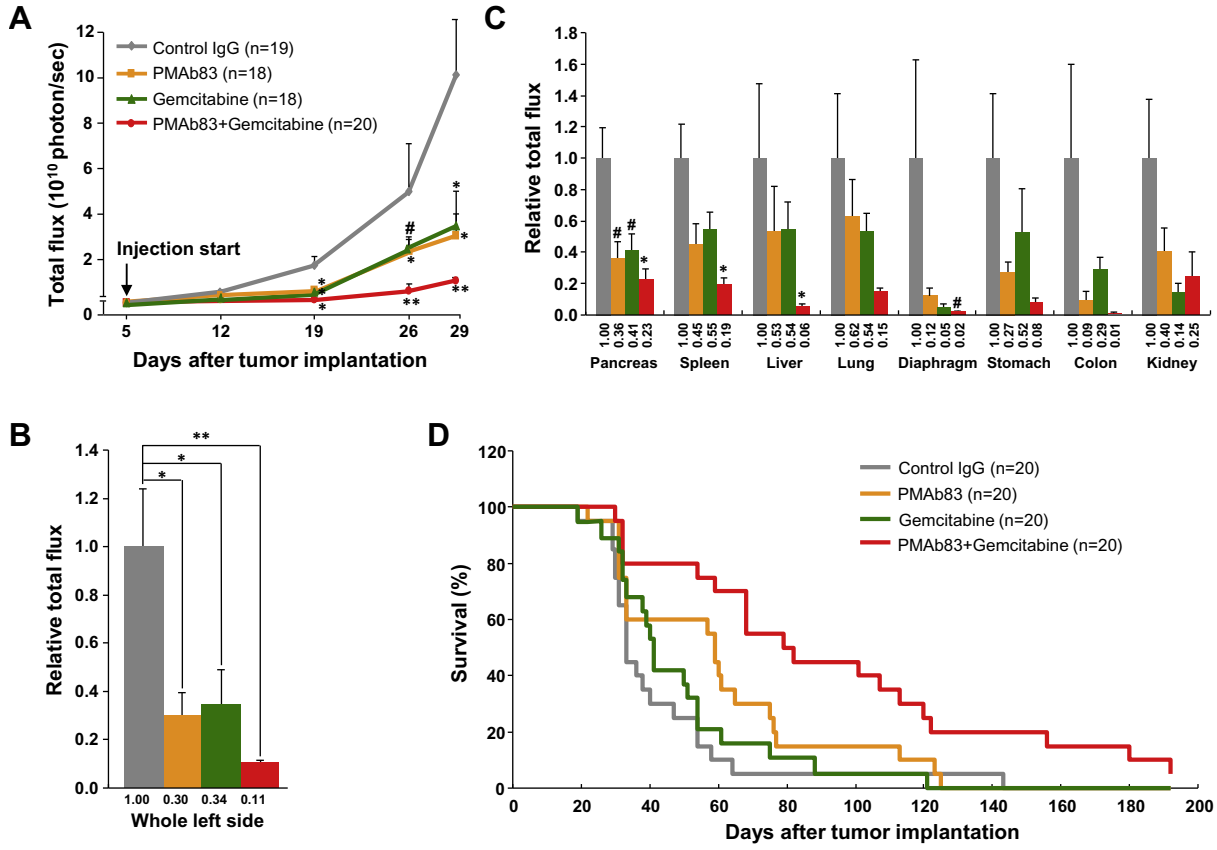

Fig. 4. PMAb83, in combination with gemcitabine, improves survival. (A) Tumor-bearing mice generated by orthotopic CFPAC-1 implantation were treated with control IgG (5 mg/kg) or PMAb83 (5 mg/kg) with or without gemcitabine (20 mg/kg) intraperitoneally twice a week. Tumor growth was presented by total flux from bioluminescence imaging in vivo. (B) At day 29 post-implantation, in vivo total flux of left side of the body was measured. (C) Mice were sacrificed to measure total flux of individual organs ex vivo. The numbers below the graph indicate relative total flux to the control IgG group. $^{\#}P < 0.05$; $^{*}P < 0.01$; $^{**}P < 0.001$. (D) Overall survival of xenograft mice was analyzed by the Kaplan–Meier method and compared using the log-rank test (combination versus control IgG, $P = 0.003$; combination versus PMAb83, $P = 0.016$; combination versus gemcitabine, $P = 0.002$).

nerve cells and extracellular matrix (ECM) [30], implying that targeting the tumor microenvironment could be a promising option for successful treatment of the disease. We recently reported a PAUF's role in promoting neo-angiogenesis and vascular permeability [23]. Our data show that PMAb83 effectively inhibits the PAUF-mediated activation of endothelial cells, markedly reducing the vascular density in xenograft tumors, which may be likely a crucial mechanism that contributes to its inhibitory effect on tumor progression and metastasis. Recent studies have demonstrated that specific blockade of the biological activities of stromal components can effectively reduce the progression of PDAC. Either the inhibition of hedgehog signaling of cancer-associated fibroblasts [31] or the enzymatic degradation of the ECM component hyaluronic acid [32] improves the delivery of therapeutic drugs and thus survival by increasing tumor vascularity. Disruption of the tumor–stromal interaction through CXCL-CXCR2 axis reduces connective tissue growth factor (CTGF) expression in fibroblasts, angiogenesis, and tumor progression [33]. Here we demonstrate that the functional inhibition of PAUF can reduce tumor growth and metastasis in orthotopic PDAC mouse models through combined mechanisms including attenuated proliferation and aggressiveness of carcinoma cells, and suppression of neo-angiogenesis. Our results provide a rationale for the development of new therapeutic strategies to treat PDAC. Future studies should include validating the efficacy of PMAb83 in more clinically-relevant experimental settings, including patient-derived xenotransplant mouse models to reflect the heterogeneity of tumors in patients.

Conflict of interest

The authors disclose no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.056.

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