Inhibition of Group 1 p21-activated Kinases Suppresses Pancreatic Stellate Cell Activation and Increases Survival of Mice with Pancreatic Cancer.

Dannel Yeo[a], Phoebe Phillips[b], Graham S. Baldwin[a], Hong He[a*], Mehrdad Nikfarjam[a*]

[a] Department of Surgery, University of Melbourne. Austin Health, Melbourne, Victoria, Australia

[b] Pancreatic Cancer Translational Research Group, Lowy Cancer Research Centre, Prince of Wales Clinical School, University of New South Wales, Sydney, NSW, Australia

*The last two authors share equal last authorship

To whom correspondence should be addressed (permanent address):

Mehrdad Nikfarjam, University of Melbourne Department of Surgery, Austin Health, Heidelberg, Victoria 3084, Australia. Email: mehrdad.nikfarjam@gmail.com

Keywords: pancreatic ductal adenocarcinoma, stroma, stroma-tumour interaction, hypoxia, fibrosis

Disclosure: There are no conflicts of interest to disclose for any of the authors.

Category: Molecular Cancer Biology

Abbreviations: PSC, Pancreatic stellate cell; hPSC, human pancreatic stellate cell; nmPSC, normal murine pancreatic stellate cell; PAK1, p21-activated kinase 1; GFAP, glial fibrillary acidic protein; αSMA, alpha-smooth muscle actin; ECM, extracellular matrix; PanIN, pancreatic intraepithelial neoplasm; EMT, epithelial to mesenchymal transition; MAPK, mitogen-activated protein kinases; NFκB, nuclear factor kappa B; HIF1α, hypoxia inducible factor 1 alpha; KO, knockout; WT, wildtype; KD, knockdown; NC, negative control; CM, conditioned media

Novelty and Impact

Pancreatic stellate cells (PSCs) are responsible for the extensive fibrosis in the stroma of pancreatic cancer where reducing PSC function may result in increased delivery of therapeutics. The role of group 1 p21-activated kinases (PAKs), specifically PAK1, has not been studied in PSCs. Here, isolated PSCs expressed PAK1, and a group1 PAK inhibitor decreased PSC function and proliferation, and increased apoptosis, by inhibiting PAK1. Depleting PAK1 in the stroma resulted in increased mouse survival. PAK1 inhibitors in combination with other therapies could increase survival of patients with pancreatic cancer by decreasing PSC function.

Abstract

Pancreatic cancer remains one of the most lethal of all solid tumours. Pancreatic stellate cells (PSCs) are primarily responsible for the fibrosis that constitutes the stroma and p21-activated kinase 1 (PAK1) may have a role in signalling pathways involving PSCs. This study aimed to examine the role of PAK1 in PSCs and in the interaction of PSCs with pancreatic cancer cells. Human PSCs were isolated using a modified outgrowth method. The effect of inhibiting PAK1 with a group 1 PAK inhibitor, FRAX597, on cell proliferation and apoptosis *in vitro* was measured by thymidine incorporation and annexin V assays, respectively. The effect of depleting host PAK1 on the survival of mice with pancreatic Pan02 cell tumours was evaluated using PAK1 knockout (KO) mice. PAK1 was expressed in isolated PSCs. FRAX597 reduced the activation of PSCs, inhibited PSC proliferation, and increased PSC apoptosis at least in partial by inhibiting PAK1 activity. The decreased expression and activity of PAK1 in PAK1 KO mice tumours was associated with increased mouse survival. These results implicate PAK1 as a regulator of PSC activation, proliferation and apoptosis. Targeting stromal PAK1 could increase therapeutic response and survival of patients with pancreatic cancer.

Introduction

Pancreatic cancer is a deadly disease with a poor survival rate and a dismal prognosis. It is the fourth leading cause of cancer-related mortality in Australia and the United States and is expected to become the second leading cause of cancer-related deaths by 2030, based on current management without significant improvements in treatment.[1] Although there have been some recent minor advances in chemotherapeutic regimens, with the addition of nab-paclitaxel to gemcitabine and the combination regimen of FOLFIRINOX improving the median survival by 2 - 11 months compared to gemcitabine alone, therapeutic response is often poor and chemoresistance is high.[2-4] One of the leading hypotheses for the poor clinical outcome is interference by the stroma, which is unique to pancreatic cancer and has been an increasing area of interest in improving outcomes for patients with pancreatic cancer.

The stroma, or desmoplastic reaction, is a dynamic milieu comprised of several different cell types including immune cells, endothelial cells, cancer-associated fibroblasts, and pancreatic stellate cells (PSCs).[5] PSCs are myofibroblast-like cells which produce the fibrosis that forms the basis of the stroma.[6-8] Although the technique for isolation of quiescent PSCs from rats and humans is well documented, there is little evidence for successful isolation from mice.[9, 10] PSCs stain positively for desmin, vimentin and glial fibrillary acidic protein (GFAP).[11] In homeostasis, PSCs are quiescent and vitamin A-containing lipid droplets are present in their cytoplasm. Upon pancreatic injury such as pancreatic cancer, PSCs lose their ability to store vitamin A and start to express alpha-smooth muscle actin (αSMA), transforming into an activated phenotype which has the ability to secrete excessive amounts of extracellular matrix (ECM) proteins.[12] In this study the conventional approach of measuring PSC activation by αSMA expression, and PSC function by expression of the ECM protein desmin, was adopted.

Targeting the stromal components of pancreatic cancer as a strategy to increase therapeutic response has thus far been unsuccessful with no clinically approved therapies.[13] Stromal depletion by administration of IPI-926, which inhibits of the hedgehog pathway, resulting in depletion of tumour-associated stromal tissue, was investigated in mouse models and resulted in increased mean vessel density with increased gemcitabine delivery and response, and a significant increase in median survival.[14] Although these results indicated that the stroma was pro-carcinogenic, more recent studies have suggested that the stroma may actually be confining and restraining the tumour.[15] Prolonged myofibroblast depletion in a transgenic mouse model resulted in more frequent precursor lesions [pancreatic intraepithelial neoplasms (PanINs)], and tumours became more metastatic and highly proliferative.[16] Also, patients with resected tumours with high stromal density had better survival after surgery and those with low stromal density had more aggressive disease.[17] A number of stroma-targeted therapies have yielded promising results in the pre-clinical setting and are currently under evaluation in clinical trials including PEGPH20,[18] which depletes hyaluronan, a component of the stromal ECM, TH-302,[19] a hypoxia-activated chemotherapeutic, modified vitamin D,[20] which targets the vitamin D receptor on PSCs resulting in stromal reprogramming, and losartan,[21] an angiotensin II type 1 receptor inhibitor which inhibits PSC activity. Understanding the biology of the stroma will be as important as understanding tumour biology, and should result in better targeted treatments with better therapeutic delivery, leading to improvements in patient survival of pancreatic cancer.

The bidirectional interaction between PSCs and pancreatic cancers is complex. PSCs can secrete a variety of paracrine factors, such as growth factors, cytokines, and chemokines, which promote pancreatic cancer growth by stimulating proliferation and inhibiting apoptosis, and stimulate invasion and metastasis by inducing an epithelial to mesenchymal transition (EMT).[22, 23] Furthermore, *in vitro* studies have found that although pancreatic

cancer has similar chemosensitivity to other cancers, patients with pancreatic cancer are less responsive to chemotherapies. These observations highlight the importance of fibrosis which results in collapsed or compressed intratumoural vasculature, and hypoxia contributing to chemotherapeutic resistance.[24] On the other hand, pancreatic cancer cells can also secrete numerous factors to activate PSCs and promote their growth.[25] The close and dynamic interaction between PSCs and pancreatic cancer cells suggests a cautious approach in translating the findings from *in vitro* studies, as PSCs may exert different effects depending on disease stage and site.

p21-activated kinases (PAKs) are a family of non-receptor serine/threonine kinases, that can mediate many effector functions from cell cycle progression and DNA transcription to cell adhesion and motility.[26] There are six PAK isoforms belonging to two groups, and PAK1, from group 1, is upregulated in a number of cancers, including pancreatic cancer.[27] The role of PAK1 has been studied in pancreatic cancer cells,[28, 29] but not, to our knowledge, in the stroma or in PSCs. However, indirect evidence suggests that PAK1 may play a role in pancreatic cancer cells upstream of both the MAPK pathway, which has been implicated in PSC activation and secretion of cytokines, and the NFκB pathway, which has been found to regulate PSC proliferation.[28, 30, 31]

FRAX597 is a small-molecule pyridopyrimidinone that targets group 1 PAKs through binding to the ATP-binding site.[32] FRAX597 inhibits PAK1 more potently than the other isoforms in the group, namely, PAK2 and PAK3.[33] FRAX597, as an inhibitor of PAK1, has been tested on a number of cancers such as skin cancer, squamous cell carcinomas, acute myeloid leukemia, non-small cell lung cancer and pancreatic cancer.[28, 33-35] FRAX597, as an inhibitor of PAK2, has also been shown to block enzyme-secretion by, and ERK1/2 activation of, pancreatic acinar cells (which only express PAK2) in response to

gastrointestinal hormones and neurotransmitters.[36] FRAX597 as a group 1 PAK inhibitor has not been tested in pancreatic stellate cells in the context of pancreatic cancer.

This study aimed to investigate the role of PAK1 in PSCs isolated from human pancreatic cancer specimens and to evaluate the potential of PAK1 as a therapeutic target in PSCs using the group 1 PAK inhibitor, FRAX597. The role of PAK1 in the interaction between PSCs and pancreatic cancer cells was investigated using orthotopic murine models in PAK1 knockout (KO) mice.

Materials and Methods

Cells and Reagents

The human PANC-1 (American Type Culture Collection, Manassas, VA) and the murine Pan02 (Division of Cancer Treatment and Diagnosis Tumour Repository, NCI, Frederick, MD) pancreatic cancer cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (fetal bovine serum; Hyclone Laboratories Inc., Scoresby, Australia). To obtain PAK1 knockdown (KD) cells, PANC-1 cells were transfected with plasmid DNAs encoding shRNA sequences to silence the PAK1 gene specifically, or with a scrambled sequence as a negative control (NC), as described previously.[28] All cells were cultured in a 37°C incubator with 5% CO_2. Cells were tested regularly for mycoplasma contamination and were not passaged more than 30 times or for more than 6 months after revival.

Human pancreatic stellate cells (hPSC) were prepared by the outgrowth method.[10] Fresh tissue was obtained from residual pancreatic adenocarcinoma specimens of patients undergoing primary surgical resection. All human samples were obtained with human ethics approval by the Austin Health Human Research Ethics Committee (H2013/04953). Briefly, tumour samples were cultured in 20% FBS in DMEM with penicillin/streptomycin. After 5-10 days, tumour fragments were transferred to a fresh culture dish and then removed after 2-4 weeks to allow PSCs to grow to confluence. Medium was changed every 3 days. The human pancreatic stellate cell line, hPSC1 (ScienCell Research Laboratories, Carlsbad, CA, USA), which had been established from human pancreas, was cultured in DMEM supplemented with 10% FBS. hPSC1 cells were found to be similar to the other isolated hPSCs in morphology and staining markers. Cells were cultured in a 37°C incubator with 5% CO_2 and not passaged more than 6 times.

Normal mouse PSCs (nmPSC) were isolated from 5 pooled pancreases using density gradient centrifugation method, based on the rat PSC isolation method.[9] Briefly, the pooled pancreases were minced and digested with protease, collagenase P and DNAse (Sigma Aldrich, Castle Hill, Australia), and the PSCs isolated from above the interface on a Nycodenz gradient (Histodenz, Sigma Aldrich). nmPSC were cultured in 20% FBS in Iscove's modified Dulbecco's medium (IMEM) with penicillin/streptomycin. Cells used for experiments were between passage 3 and 6.

PSC purity was determined by immunocytochemistry for αSMA (ab32575), GFAP (ab7260) and desmin (ab32362), as well as morphology (spindle-shaped cells with cytoplasmic extensions). Only cells that were negative for cytokeratin 19 (CK19; ab52625) were used, to exclude tumour contamination. All antibodies used were from Abcam (Cambridge, MA, USA).

FRAX597 was purchased from SYNthesis (Parkville, Australia), reconstituted in DMSO and stored at 4°C. The resulting DMSO concentration was less than 5% for *in vitro* studies.

Immunohistochemistry (IHC)

For PAK1 IHC, human and murine sample sections were incubated with 3% hydrogen peroxide in methanol for 10 min at room temperature to quench endogenous peroxidase activity. Antigen were retrieved by incubation in 10 mM citrate buffer and blocked in 5% horse serum. Sections were incubated with antibody against PAK1 (1:200) (sc882; Santa Cruz Biotechnology, Dallas, TX) or IgG (sc2027) and visualised using an ENvision Plus polymer-based detection kit (Dako, Botany, Australia). The slides were then counter-stained and images were taken with a NIKON Coolscope (Coherent Scientific, Hilton, Australia).

Immunofluorescence

Human sample sections were quenched, blocked and antigen retrieved as described above. Sections were incubated with rabbit antibody against PAK1 (1:100) (sc882; Santa Cruz Biotechnology), and mouse antibody against αSMA (1:300) (A5228; Sigma Aldrich) then incubated with secondary Alexa Fluor 488 anti-rabbit and Alexa Fluor 546 anti-mouse antibodies. The slides were incubated in DAPI and images were taken with a fluorescence microscope (Leica Biosystems, Mt Waverley, Australia).

Western Blot

Proteins were detected with antibodies against phospho-PAK1 (2606; Cell Signalling Technology, Arundel, Australia), PAK1 (2602; Cell Signalling Technology), desmin (sc14026; Santa Cruz Biotechnology), αSMA (Abcam), cyclin D1 (2978; Cell Signalling Technology), HIF1α (610958; hypoxia-inducible factor 1α; BD Biosciences, North Ryde, Australia), and GAPDH (2118; Cell Signalling Technology). HIF1α expression was determined in cells cultured under normoxia or hypoxia (1% O_2). For conditioned media (CM) experiments, PANC-1 KD cells were cultured in 6-well dishes for 48 hours. Media were collected, filtered and directly added to PSCs for 48 hours before the PSCs were lysed and protein collected.

Cell Proliferation, Apoptosis, and Migration

Cell proliferation was measured by ^{3}H-thymidine incorporation as previously described.[29]

Cell apoptosis was assessed using the annexin V-FITC apoptosis detection kit (BioVision, Milpilas, CA, USA). Cells were analysed by flow cytometry, after 24 hour treatment with FRAX597 and stained with annexin V-FITC reagent and/or propidium iodide (PI).

Cell migration and invasion was measured using the Transwell Boyden Chamber assay. PSCs were seeded in the lower chamber at varying ratios to cancer cells. PANC-1 or PANC-1

PAK1 KD cells were seeded in the upper chamber of the inserts (Falcon®, 8μm pore size; Corning, NY, USA). Cells that had attached to the underside of the membrane were fixed and counted using a NIKON Coolscope (Coherent Scientific, Hilton, Australia).

Murine Orthotopic Pancreatic Cancer Model

All mice experiments were approved by the Austin Health Animal Research Ethics Committee (A2013/04898). Pan02 cells were implanted orthotopically in the head of the pancreas of 17 C57Bl/6 mice (WT) and 22 C57Bl/6 PAK1 KO ($PAK1^{-/-}$) mice, as previously described.[37] Mice were monitored for health score for up to 30 days and euthanased when a poor health score was reached. To obtain sufficient numbers, 4 studies were completed. A collated Kaplan-Meier survival curve was plotted, and the data were analysed together using stratified Cox regression analysis (SPSS; IBM, New York, NY). 3 mice tumours from WT and PAK1 KO mice, that were euthanased at the same time point, were isolated, cut into blocks and fixed for hematoxylin and eosin (H&E) staining and for immunohistochemistry, or snap frozen for western blot analysis.

Statistical Analysis

All values are expressed as means $\pm$ standard error. Experiments were done in duplicate and data collated from three independent experiments. Results were analysed using Student's t-test or one-way ANOVA (SPSS). Differences between two means with $p < 0.05$ were considered significant.

Results

PAK1 is expressed in pancreatic cancer stroma and in isolated pancreatic stellate cells

PAK1 staining was observed in the stromal component of human pancreatic cancer specimens (figure 1A&B), with the negative control showing no positive staining (supplementary figure S1). Stromal PAK1 staining could be observed surrounding PanIN lesions. PAK1 was expressed in activated stellate cells with the co-localised staining of PAK1 and the activated stellate cell marker, αSMA (figure 1B). An example of the individual staining is shown in supplementary figure S2. Myofibroblast-like cells were isolated from these specimens and confirmed to be PSCs by their positive staining for desmin, GFAP, and αSMA with strong PAK1 staining in the cytoplasmic compartment (figure 1C). PSCs were successfully isolated from 4 different human pancreatic cancer specimens, and the expression of PAK1, desmin and αSMA was compared by Western blotting to the human pancreatic stellate cell line hPSC1. All cell lines expressed PAK1, αSMA and desmin, although the relative amounts varied (figure 1D). PAK1 expression positively correlated with the expression of αSMA, but not desmin. The data indicate that PAK1 is present in PSCs and suggests that PAK1 activity may be important in pancreatic cancer.

FRAX597 inhibited stellate cell activation, activity and proliferation, and increased apoptosis

The group 1 PAK inhibitor, FRAX597, significantly decreased PAK1 expression and activity (figure 2A) and significantly decreased αSMA and desmin expression at 1μM and 2μM (figure 2B) in the hPSC1 cell line, although in the case of αSMA the inhibition did not appear to be dose dependent. Presumably at the higher concentrations of FRAX597 there is a compensatory upregulation of αSMA expression by PAK1-independent pathways. FRAX597 decreased cyclin D1 expression (figure 2B) and inhibited proliferation in a dose-dependent manner with an IC_{50} of 0.61 $\pm$ 0.28 μM (figure 2C). At 1 μM, FRAX597 induced PSC

apoptosis with a 3.8-fold increase in apoptotic cells (from 2.7% in untreated cells to 10.2% in the FRAX597-treated cells) (figure 2D, supplementary figure S3). These results indicated that inhibition of PAK1 by FRAX597 inhibited the activation (as measured by αSMA expression) of PSCs, decreased proliferation by inhibition of cyclin D1 expression, and increased apoptosis.

Hypoxia increased expression and activity of PAK1 in PSCs

We have previously reported that knockdown of PAK1 significantly reduced the increase in HIF1α expression observed in pancreatic cancer cell lines on exposure to hypoxia.[28] As this observation suggested that PAK1 may play a role in the resistance of pancreatic cancer to hypoxia through regulation of HIFα, the involvement of PAK1 in the response of PSCs to hypoxia was investigated. PAK1 expression and activity, and αSMA expression, were significantly increased in the hPSC1 cell line under hypoxia (figure 3A). At a concentration of 1μM, FRAX597 significantly inhibited PAK1 expression and activity, and αSMA expression under both normoxia and hypoxia. HIF1α expression was induced by hypoxia and the induction was significantly inhibited by FRAX597 at 1μM under both normoxia and hypoxia (figure 3B). These results suggested a positive relationship between the expression and activity of PAK1, and the activation of PSCs and HIF1α expression, in response to the switch from normoxia to hypoxia.

PAK1 KO increased survival of mice with pancreatic cancer

Quiescent normal murine pancreatic stellate cells (nmPSCs) were successfully isolated from PAK1 KO and WT mice, and shown to express desmin and GFAP (supplementary figure S4A). As expected, the PAK1 KO nmPSCs did not express any PAK1 staining by immunocytochemistry or PAK1 protein expression and activity by western blot but were similar compared to WT based on cell morphology (figure 4A). Growth of PAK1 KO

nmPSCs was significantly less than WT nmPSCs at 72 hours (figure 4B). Cells of the PAK1-positive mouse pancreatic cancer cell line Pan02 were injected into the head of the pancreas of PAK1 KO or WT mice. After 30 days, PAK1 expression and activity in the tumours from PAK1 KO mice was significantly less than in the tumours from PAK1 WT mice (figure 4C). PAK1 expression and activity levels in the tumours of PAK1 WT mouse were not significantly different compared to Pan02 cells (supplementary figure S4B). Expression of αSMA, desmin and collagen at the endpoint was similar between tumours from PAK1 KO and WT mice (supplementary figure 3C). Survival of PAK1 KO mice was significantly greater than PAK1 WT mice, with 44% of mice surviving to the 30 day endpoint compared to 8% of mice in the WT group (figure 4D). Using WT mice as a reference, PAK1 KO mice had a hazard ratio of 0.41 (0.18 ± 0.94) with a p-value of 0.035 (table 1). This result is in agreement with the prolongation of survival observed in mice treated with FRAX597 in the orthotopic pancreatic cancer model.[28] The results confirmed that PAK1 is involved in PSC proliferation, with a decrease observed in PAK1 KO nmPSCs, and indicated that depletion of PAK1 in pancreatic stroma containing PSCs reduced PAK1 expression and activity in the tumours, leading to increased mouse survival.

PAK1 knockdown in pancreatic cancer cells negatively regulated PSCs and the interaction between PSCs and pancreatic cancer cells

To test the effect of modulation of PAK1 in tumour cells on surrounding PSCs, cells of the human pancreatic stellate cell line, hPSC1, were incubated with media conditioned either by a clone of the human pancreatic cancer cell line PANC-1 which had been transfected with a scrambled control sequence, or by PANC1 clones in which PAK1 expression had been reduced by shRNA knockdown. PAK1 expression and activity were significantly decreased in hPSC1 cells incubated with conditioned medium from either knockdown clone (figure 5A). Desmin expression was also significantly decreased in hPSC1 cells incubated with

conditioned medium from either knockdown clone, but αSMA was unchanged (figure 5B). The results suggested that a reduction of PAK1 expression in pancreatic cancer cells suppressed the expression and activity of PAK1 in PSCs, and hence indicated that PAK1 was important in signalling, not only from pancreatic stroma containing PSCs to tumour cells, but also from tumour cells to PSCs.

This conclusion was confirmed by investigation of the effect of PSCs on migration/invasion of PANC1 clones in which PAK1 expression had been reduced by shRNA knockdown. We have previously reported that PAK1 knockdown significantly reduced proliferation of both PANC1 and MiaPaCa2 cells.[28] In this study a similar inhibitory effect was observed on PANC1 migration/invasion (figure 5D). Increasing the ratio of stellate cells to tumour cells to 50:1 significantly increased PANC-1 cell migration/invasion (figure 5C). At the same ratio of 50:1, PSCs significantly enhanced the migration/invasion of PANC-1 NC cells, but not of the PAK1 KD PANC-1 cells (figure 5D). This result strengthened the conclusion that the bidirectional signalling traffic between tumour cells and PSCs involves PAK1 in both directions.

Discussion

This is the first study, to our knowledge, to identify a role for PAK1 in the pancreatic stroma. Expression of PAK1 was detected in the pancreatic stroma of human pancreatic cancer specimens including stroma surrounding PanINs (figure 1A). PanIN-associated stroma has been identified with increasing fibrosis correlated with increasing PanIN grade.[13] This observation suggests that PAK1 is involved in the stroma of pre- and established pancreatic cancer and could be a potential therapeutic target. However, fibrosis can also be due to chronic pancreatitis which is a risk factor for pancreatic cancer and often diagnosed concurrently.[38] The origin of fibrosis may be an interesting area of study as PAK1 may be used as a diagnostic or prognostic marker. Although there are many cell types within the stroma, PSCs were chosen for further study as they are primarily responsible for the extensive fibrosis observed.

PAK1 expression in isolated human PSCs correlated with the expression of αSMA, but not with the expression of desmin. This result suggests that PAK1 plays a role in the activation of stellate cells in pancreatic cancer where more activated stellate cells (higher αSMA expression) had higher PAK1 expression. This can also be observed in figure 1B where PAK1 (green) is expressed in activated stellate cells (red) resulting in co-staining (indicated by the arrows). Although, a small sample size, the tumour sample from the isolated PSC which had high PAK1 did have more co-localised staining compared to the tumour sample from the isolated PSC which had low PAK1 (data not shown). Examination of the histopathological and patient data (data not shown) did not reveal any correlation with the level of PAK1 expression in hPSCs, however, larger numbers are needed to strengthen this conclusion. Moreover, the involvement of PAK1 in other stromal cell types such as immune cells and in neuroendocrine cells warrants further investigation.[39, 40]

FRAX597 switched stellate cells to a more quiescent phenotype by inhibition of the activation and function of hPSCs (figure 2B), inhibited stellate cell proliferation (figure 2C) by reducing the expression of cyclin D1, and promoted apoptosis (figure 2D). FRAX597 is selective for PAK1, but also inhibits PAK2 and PAK3, with little effect on PAK4-6.[32] FRAX597 also targets other kinases including RET, YES1, TEK, and CSF1R.[32] Although the ability of FRAX597 to inhibit PAK1 was confirmed (figure 2A), and there is no evidence to suggest that either PAK2 or PAK3 plays any role in pancreatic cancer, their involvement and the involvement of the other targets of FRAX597 should not be disregarded and warrants further investigation.[41, 42] Attempts to confirm the inhibitor studies by PAK1 knockdown were uniformly unsuccessful (data not shown). Stable PAK1 knockdown was undertaken in the hPSC1 cell line, but the transfected cells did not grow under selection. Transient transfection was also attempted but was only done successfully once before the cells transformed. Nevertheless, our results are consistent with the hypothesis that PAK1 is involved in multiple pathways including those regulating PSC activation, cell cycle, apoptosis and proliferation. The depletion of stellate cells suggested by the induction of apoptosis in figure 2D may result in more aggressive tumours, but a decrease in stellate cell activation and an inhibition of proliferation was also observed, suggestive of a reprogramming of the stellate cells to their inactive state.[5] Further studies are needed to confirm if this dual strategy results in improved outcomes for pancreatic cancer patients.

Hypoxia increased PAK1 expression and activity and activated stellate cells (figure 3A). Pancreatic tumours are known to be highly hypoxic and hypoxia has been found to induce PSC activity, migration, and activation.[43-45] The expression of HIF1α, a master regulator of the cellular response to hypoxia, was increased under hypoxia, and we have previously reported that the increase in HIF1α expression is downstream from PAK1 in pancreatic tumour cells.[28] Reactive oxygen species (ROS) under hypoxia have been suggested to

activate PSCs through stabilisation of HIF1α, further implicating PAK1 in the activation of PSCs.[46] The data suggest that PAK1 may also be induced to increase HIF1α as a survival mechanism for PSCs under a hypoxic challenge.[28] Under hypoxia, FRAX597 significantly decreased PSC activation and HIF1α, switching PSCs to the quiescent phenotype and reversing the effect of hypoxia-related survival. These observations further implicate PAK1 in key PSC mechanisms and highlight PAK1's therapeutic potential.

nmPSCs from PAK1 KO mice were normal, based on cell morphology, but had impaired proliferative ability (figure 4B), and this decrease correlated with decreased activity and expression of PAK1 in the pancreatic tumours in PAK1 KO mouse (figure 4C) and increased mouse survival (figure 4D). The fact that proliferation of nmPSCs from PAK1 KO mice was reduced highlighted PAK1's role in PSC proliferation and confirmed the effect of FRAX597 *in vitro* (figure 2C). nmPSCs from PAK1 KO mice had normal cell morphology (figure 4A) and were successfully isolated, indicating that PAK1 is not involved in the development of stellate cells and is comparable to nmPSCs from WT mice pre-tumour induction. The resulting improvement in survival can be attributed to the disruption in PAK1 signalling in the stromal of PAK1 KO mice. The pancreatic tumours grown in PAK1 KO mice had reduced PAK1 staining although the tumour cells were originally PAK1 positive at induction. This observation suggests that the stroma can regulate PAK1 expression in the tumours, possibly through the involvement of negative regulators of PAK1. Although the exact mechanism of interaction that resulted in the deregulation of tumoural PAK1 is unclear, the observed reduction does highlight the importance of stromal PAK1, the complex interaction between PSCs and the tumour, and the potential therapeutic benefit of targeting PAK1 in the stroma to increase pancreatic cancer survival.

PAK1 plays an important role in the interaction between PSCs and tumour cells. PAK1 activity and expression in stellate cells was reduced (figure 5A) when PSCs were cultured

with the media conditioned by tumour cells in which PAK1 had been knockdown by transfection with shRNA. This result suggests that PAK1-dependent pathways in the tumour control the secretion of factors that regulate the expression of PAK1 in the stellate cells. Together with the *in vivo* results, this observation suggests that targeting PAK1 expression in either PSCs or tumour cells results in deregulation of PAK1 in the other cell type. Knockdown of tumoural PAK1 also resulted in a decrease in stellate cell function. Although this result shows that PAK1 in the tumours is responsible for the secretion of factors which regulate stellate function, PAK1 is unlikely to be the main regulator as only a modest decrease was observed and many other pathways, which have been well studied, are also involved.[30] The observation that there was no significant change in stellate cell activation after treatment with the media conditioned by PAK1 KD tumour cells suggests, either that tumoural PAK1-dependent secretion may not be involved in the deactivation/activation of the stellate cells, or that the residual low PAK1 is able to stimulate the production of factors that can maintain the stellate cell's activated state. Although this study only examines the unidirectional interaction, it does implicate the involvement of PAK1 pathways in the tumour in controlling the secretion of factors influencing PAK1 regulation and stellate cell activity.

Stellate cell-driven migration and invasion of tumours are PAK1-dependent (figure 5C-D). Increasing stellate-tumour cell ratios resulted in increased migration and invasion of tumour cells with a 3-fold increase at a ratio of 50 (figure 5C). In the absence of stellate cells, PAK1 KD tumour cells had reduced migration and invasion. When incubated with stellate cells, migration and invasion of PAK1 KD tumour cells was severely impaired, compared to controls (figure 5D). This result suggests that stellate cells secrete factors that regulate the migration and invasion of tumour cells and must involve PAK1 pathways in the tumour. Hence patients with tumours with low PAK1 expression may not have more aggressive tumours with stromal depletion therapy, as initially suggested in a study involving

myofibroblast depletion in a mouse model.[16] Nevertheless, the results presented here support the use of a PAK1 inhibitor to target the tumour cells, both directly and through indirect targeting of stromal PAK1, to impair metastasis and increase patient survival.

Conclusion

PAK1 is expressed in the stroma of pancreatic cancer specimens, as well as in pancreatic stellate cells isolated from human pancreatic cancer patients. Hypoxia increased PAK1 activity and expression in the human pancreatic stellate cell line, hPSC1. The group 1 PAK inhibitor, FRAX597, inhibited proliferation by reducing the expression of cyclin D1. FRAX597 also increased apoptosis, and reduced hPSC1 activation, and HIF1α expression. In the interaction of stellate cells and tumour cells, depletion of stromal PAK1 in PAK1 KO mice resulted in reduced activity and expression of PAK1 in pancreatic tumours generated by inoculation of murine Pan02 cells, which were originally PAK1 positive, and increased mouse survival. This result suggests that an indirect strategy to target the pancreatic tumours by modulation of the stroma would be effective. The complex and bidirectional regulation of PAK1 between stellate cells and tumour cells was further illustrated by the observation that regulation of PAK1 in stellate cells was also dependent on PAK1 expression in the tumour cells. Stellate cell-driven migration and invasion of tumour cells was found to utilise PAK1 pathways since PAK1 KD tumour cells had impaired migration and invasion in the presence of stellate cells. In conclusion, PAK1-directed therapy has the potential not only to disrupt the pancreatic cancer stroma, by switching the stellate cells to a quiescent phenotype and hence reducing the associated fibrosis, but also to reduce pancreatic tumour metastasis and hence increase survival in patients with pancreatic cancer.

Acknowledgements

This work was supported by grants from the National Health and Medical Research Council of Australia (1020983, GSB); the Pancare Foundation (GSB, MN); the Austin Hospital Medical Research Foundation (MN); and the Sir Edward Dunlop Foundation (MN). PP is supported by a National Health and Medical Research Council (NHMRC) CDF1 Fellowship. DY is supported by Australian Rotary Health (The Ian Loxton Pancreatic Cancer Research PhD Scholarship).

References

1. Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer research* 2014;**74**: 2913-21.

2. Long J, Zhang Y, Yu X, Yang J, LeBrun DG, Chen C, Yao Q, Li M. Overcoming drug resistance in pancreatic cancer. *Expert opinion on therapeutic targets* 2011;**15**: 817-28.

3. Suker M, Beumer BR, Sadot E, Marthey L, Faris JE, Mellon EA, El-Rayes BF, Wang-Gillam A, Lacy J, Hosein PJ, Moorcraft SY, Conroy T, et al. FOLFIRINOX for locally advanced pancreatic cancer: a systematic review and patient-level meta-analysis. *The Lancet Oncology* 2016.

4. Von Hoff DD, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, Seay T, Tjulandin SA, Ma WW, Saleh MN, Harris M, Reni M, et al. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *The New England journal of medicine* 2013;**369**: 1691-703.

5. Neesse A, Algul H, Tuveson DA, Gress TM. Stromal biology and therapy in pancreatic cancer: a changing paradigm. *Gut* 2015;**64**: 1476-84.

6. Hwang RF, Moore T, Arumugam T, Ramachandran V, Amos KD, Rivera A, Ji B, Evans DB, Logsdon CD. Cancer-associated stromal fibroblasts promote pancreatic tumor progression. *Cancer research* 2008;**68**: 918-26.

7. Vonlaufen A, Joshi S, Qu C, Phillips PA, Xu Z, Parker NR, Toi CS, Pirola RC, Wilson JS, Goldstein D, Apte MV. Pancreatic stellate cells: partners in crime with pancreatic cancer cells. *Cancer research* 2008;**68**: 2085-93.

8. Vonlaufen A, Phillips PA, Xu Z, Goldstein D, Pirola RC, Wilson JS, Apte MV. Pancreatic stellate cells and pancreatic cancer cells: an unholy alliance. *Cancer research* 2008;**68**: 7707-10.

9. Apte MV, Haber PS, Applegate TL, Norton ID, McCaughan GW, Korsten MA, Pirola RC, Wilson JS. Periacinar stellate shaped cells in rat pancreas: identification, isolation, and culture. *Gut* 1998;**43**: 128-33.

10. Bachem MG, Schneider E, Gross H, Weidenbach H, Schmid RM, Menke A, Siech M, Beger H, Grunert A, Adler G. Identification, culture, and characterization of pancreatic stellate cells in rats and humans. *Gastroenterology* 1998;**115**: 421-32.

11. Omary MB, Lugea A, Lowe AW, Pandol SJ. The pancreatic stellate cell: a star on the rise in pancreatic diseases. *The Journal of clinical investigation* 2007;**117**: 50-9.

12. Erkan M, Adler G, Apte MV, Bachem MG, Buchholz M, Detlefsen S, Esposito I, Friess H, Gress TM, Habisch HJ, Hwang RF, Jaster R, et al. StellaTUM: current consensus and discussion on pancreatic stellate cell research. *Gut* 2012;**61**: 172-8.

13. Erkan M, Hausmann S, Michalski CW, Fingerle AA, Dobritz M, Kleeff J, Friess H. The role of stroma in pancreatic cancer: diagnostic and therapeutic implications. *Nature reviews Gastroenterology & hepatology* 2012;**9**: 454-67.

14. Olive KP, Jacobetz MA, Davidson CJ, Gopinathan A, McIntyre D, Honess D, Madhu B, Goldgraben MA, Caldwell ME, Allard D, Frese KK, Denicola G, et al. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science* 2009;**324**: 1457-61.

15. Rhim AD, Oberstein PE, Thomas DH, Mirek ET, Palermo CF, Sastra SA, Dekleva EN, Saunders T, Becerra CP, Tattersall IW, Westphalen CB, Kitajewski J, et al. Stromal elements act to restrain, rather than support, pancreatic ductal adenocarcinoma. *Cancer cell* 2014;**25**: 735-47.

16. Ozdemir BC, Pentcheva-Hoang T, Carstens JL, Zheng X, Wu CC, Simpson TR, Laklai H, Sugimoto H, Kahlert C, Novitskiy SV, De Jesus-Acosta A, Sharma P, et al.

Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival. *Cancer cell* 2014;**25**: 719-34.

17. Torphy RJ, Volmar KE, Naim R, Cader SR, Johansen JS, Hollingsworth MA, Collison EA, Yeh JJ. Quantification of tumor stroma as a biomarker in pancreatic adenocarcinoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2015;**33**.

18. Hingorani SR, Harris WP, Seery TE, Zheng L, Sigal D, Hendifar AE, Braiteh FS, Zalupski M, Baron AD, Bahary N, Wang-Gillam A, LoConte NK, et al. Interim results of a randomized phase II study of PEGPH20 added to nab-paclitaxel/gemcitabine in patients with stage IV previously untreated pancreatic cancer Journal of Clinical Oncology, vol. 34, 2016:439.

19. Borad MJ, Reddy SG, Bahary N, Uronis HE, Sigal D, Cohn AL, Schelman WR, Stephenson J, Jr., Chiorean EG, Rosen PJ, Ulrich B, Dragovich T, et al. Randomized Phase II Trial of Gemcitabine Plus TH-302 Versus Gemcitabine in Patients With Advanced Pancreatic Cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2015;**33**: 1475-81.

20. Sherman MH, Yu RT, Engle DD, Ding N, Atkins AR, Tiriac H, Collisson EA, Connor F, Van Dyke T, Kozlov S, Martin P, Tseng TW, et al. Vitamin D receptor-mediated stromal reprogramming suppresses pancreatitis and enhances pancreatic cancer therapy. *Cell* 2014;**159**: 80-93.

21. Chauhan VP, Martin JD, Liu H, Lacorre DA, Jain SR, Kozin SV, Stylianopoulos T, Mousa AS, Han X, Adstamongkonkul P, Popovic Z, Huang P, et al. Angiotensin inhibition enhances drug delivery and potentiates chemotherapy by decompressing tumour blood vessels. *Nature communications* 2013;**4**: 2516.

22. Haqq J, Howells LM, Garcea G, Metcalfe MS, Steward WP, Dennison AR. Pancreatic stellate cells and pancreas cancer: current perspectives and future strategies. *European journal of cancer* 2014;**50**: 2570-82.

23. Pothula SP, Xu Z, Goldstein D, Pirola RC, Wilson JS, Apte MV. Key role of pancreatic stellate cells in pancreatic cancer. *Cancer letters* 2015.

24. McCarroll JA, Naim S, Sharbeen G, Russia N, Lee J, Kavallaris M, Goldstein D, Phillips PA. Role of pancreatic stellate cells in chemoresistance in pancreatic cancer. *Frontiers in physiology* 2014;**5**: 141.

25. Moir JA, Mann J, White SA. The role of pancreatic stellate cells in pancreatic cancer. *Surgical oncology* 2015;**24**: 232-8.

26. Rane CK, Minden A. P21 activated kinases: structure, regulation, and functions. *Small GTPases* 2014.

27. Yeo D, He H, Baldwin GS, Nikfarjam M. The role of p21-activated kinases in pancreatic cancer. *Pancreas* 2015;**44**: 363-9.

28. Yeo D, He H, Patel O, Lowy AM, Baldwin GS, Nikfarjam M. FRAX597, a PAK1 inhibitor, synergistically reduces pancreatic cancer growth when combined with gemcitabine. *BMC cancer* 2016;**16**: 1-12.

29. Yeo D, Huynh N, Beutler JA, Christophi C, Shulkes A, Baldwin GS, Nikfarjam M, He H. Glaucarubinone and Gemcitabine Synergistically Reduce Pancreatic Cancer Growth via Down-Regulation of P21-Activated Kinases. *Cancer letters* 2014;**346**: 264-72.

30. Masamune A, Shimosegawa T. Signal transduction in pancreatic stellate cells. *Journal of gastroenterology* 2009;**44**: 249-60.

31. Yoshida S, Yokota T, Ujiki M, Ding XZ, Pelham C, Adrian TE, Talamonti MS, Bell RH, Jr., Denham W. Pancreatic cancer stimulates pancreatic stellate cell proliferation and TIMP-1 production through the MAP kinase pathway. *Biochemical and biophysical research communications* 2004;**323**: 1241-5.

32. Licciulli S, Maksimoska J, Zhou C, Troutman S, Kota S, Liu Q, Duron S, Campbell D, Chernoff J, Field J, Marmorstein R, Kissil JL. FRAX597, a Small Molecule Inhibitor of the p21-activated Kinases, Inhibits Tumorigenesis of Neurofibromatosis Type 2 (NF2)-associated Schwannomas. *The Journal of biological chemistry* 2013;**288**: 29105-14.

33. Chow HY, Jubb AM, Koch JN, Jaffer ZM, Stepanova D, Campbell DA, Duron SG, O'Farrell M, Cai KQ, Klein-Szanto AJ, Gutkind JS, Hoeflich KP, et al. p21-Activated kinase 1 is required for efficient tumor formation and progression in a Ras-mediated skin cancer model. *Cancer research* 2012;**72**: 5966-75.

34. Pandolfi A, Stanley RF, Yu Y, Bartholdy B, Pendurti G, Gritsman K, Boultwood J, Chernoff J, Verma A, Steidl U. PAK1 is a therapeutic target in acute myeloid leukemia and myelodysplastic syndrome. *Blood* 2015;**126**: 1118-27.

35. Mortazavi F, Lu J, Phan R, Lewis M, Trinidad K, Aljilani A, Pezeshkpour G, Tamanoi F. Significance of KRAS/PAK1/Crk pathway in non-small cell lung cancer oncogenesis. *BMC cancer* 2015;**15**: 381.

36. Nuche-Berenguer B, Ramos-Alvarez I, Jensen RT. The p21-activated kinase, PAK2, is important in the activation of numerous pancreatic acinar cell signaling cascades and in the onset of early pancreatitis events. *Biochimica et biophysica acta* 2016;**1862**: 1122-36.

37. Nikfarjam M, Yeo D, He H, Baldwin G, Fifis T, Costa P, Tan B, Yang E, Wen SW, Christophi C. Comparison of Two Syngeneic Orthotopic Murine Models of Pancreatic Adenocarcinoma. *Journal of investigative surgery : the official journal of the Academy of Surgical Research* 2013;**26**: 352-9.

38. Whitcomb DC. Inflammation and Cancer V. Chronic pancreatitis and pancreatic cancer. *American journal of physiology Gastrointestinal and liver physiology* 2004;**287**: G315-9.

39. Kelly ML, Chernoff J. Mouse models of PAK function 2012;**2**: 84-8.

40. Gasman S, Chasserot-Golaz S, Malacombe M, Way M, Bader MF. Regulated exocytosis in neuroendocrine cells: a role for subplasmalemmal Cdc42/N-WASP-induced actin filaments. *Molecular biology of the cell* 2004;**15**: 520-31.

41. Kumar R, Gururaj AE, Barnes CJ. p21-activated kinases in cancer. *Nature reviews Cancer* 2006;**6**: 459-71.

42. He H, Baldwin GS. p21-activated kinases and gastrointestinal cancer. *Biochimica et biophysica acta* 2013;**1833**: 33-9.

43. Masamune A, Kikuta K, Watanabe T, Satoh K, Hirota M, Shimosegawa T. Hypoxia stimulates pancreatic stellate cells to induce fibrosis and angiogenesis in pancreatic cancer. *American journal of physiology Gastrointestinal and liver physiology* 2008;**295**: G709-17.

44. Erkan M, Reiser-Erkan C, Michalski CW, Deucker S, Sauliunaite D, Streit S, Esposito I, Friess H, Kleeff J. Cancer-stellate cell interactions perpetuate the hypoxia-fibrosis cycle in pancreatic ductal adenocarcinoma. *Neoplasia* 2009;**11**: 497-508.

45. Rebours V, Albuquerque M, Sauvanet A, Ruszniewski P, Levy P, Paradis V, Bedossa P, Couvelard A. Hypoxia pathways and cellular stress activate pancreatic stellate cells: development of an organotypic culture model of thick slices of normal human pancreas. *PloS one* 2013;**8**: e76229.

46. Lei J, Huo X, Duan W, Xu Q, Li R, Ma J, Li X, Han L, Li W, Sun H, Wu E, Ma Q. alpha-Mangostin inhibits hypoxia-driven ROS-induced PSC activation and pancreatic cancer cell invasion. *Cancer letters* 2014;**347**: 129-38.

Table 1. Survival of PAK1 knockout ($PAK1^{-/-}$) mice was greater than wild type (WT) mice in an orthotopic murine pancreatic cancer model.

Group	Hazard Ratio	95% CI	p value
WT	1.0 (ref)		
$PAK1^{-/-}$	0.41	0.18 ± 0.94	0.035

Cells of the PAK1-positive mouse pancreatic cancer cell line Pan02 were injected into the head of the pancreas of PAK1 KO or WT mice, and survival was followed over the next 30 days. The overall statistics for the stratified Cox regression analysis were: χ^2 (1) = 4.7, p = 0.031.

Figure Legends

Figure 1: PAK1 is expressed in the stroma of human pancreatic cancer specimens and in isolated pancreatic stellate cells.

Representative images show the varying levels of PAK1 staining in the microenvironment of human pancreatic cancer specimens at x200 magnification (A). Representative images show the co-staining of PAK1 (green) and activated stellate cells (red, staining αSMA) indicated by the orange arrow at x100 (left image) and x200 (right image) magnification (B). Nuclear staining by DAPI is shown in blue and a representative image with the individual colours shown in supplementary figure S2. The purity of pancreatic stellate cells (PSC), isolated from human pancreatic cancer specimens, was confirmed by morphology, and by immunocytochemical staining for desmin, GFAP (glial fibrillary acidic protein) and αSMA (alpha-smooth muscle actin) (C). The human pancreatic stellate cell line hPSC1 and PSC isolated from 4 different human pancreatic cancer specimens expressed varying levels (ratio to GAPDH) of PAK1, desmin and αSMA as detected by western blot (D). PAK1 expression correlated positively with αSMA expression, but not with desmin expression. The data represent mean ± SEM, summarised from three independent experiments.

Figure 2: The group 1 PAK inhibitor, FRAX597, decreased PAK1 activity and expression and PSC activation and activity, inhibited PSC proliferation, and increased apoptosis.

FRAX597, a group 1 PAK inhibitor, reduced the activity and expression of PAK1 (A), reduced stellate cell activation (αSMA expression) and desmin expression, and decreased expression of the cell cycle promoter, cyclin D1 (B) after 24 hour treatment on the hPSC1 cell line. FRAX597 inhibited proliferation after 24 hours treatment in a dose dependent manner (C) and increased apoptosis (D). The data (mean ± SEM, summarised from three

independent experiments) are presented as a percentage to untreated cells, except for pPAK1 expression, which is presented as a percentage of the untreated PAK1 value. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to untreated cells.

Figure 3: Hypoxia increased PAK1 expression and activity and FRAX597 decreased the expression of αSMA and HIF1α.

PAK1 expression and activity and αSMA expression were increased under hypoxia but were reduced after treatment with 1μM FRAX597 under both normoxia and hypoxia (A) in the hPSC1 cell line. The group 1 PAK inhibitor, FRAX597 also reduced HIF1α expression under both normoxia and hypoxia (D). The data (mean ± SEM, summarised from three independent experiments) are presented as a percentage of the corresponding value for untreated normoxic cells, except for pPAK1 expression, which is presented as a percentage of the PAK1 value for untreated normoxic cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to normoxic untreated cells. # $p < 0.05$; ## $p < 0.01$ as indicated in the figure.

Figure 4: In PAK1 KO mice, growth of quiescent PSCs was reduced, PAK1 expression in pancreatic tumours was decreased, and survival was increased, compared to PAK1 WT mice.

Quiescent murine stellate cells (nmPSC) were successfully isolated from pancreases of PAK1 knockout (KO) mice and shown by cell morphology to be normal compared to the wildtype (WT) mice although they express negligible amounts of PAK1 by immunocytochemistry and western blot (ratio to GADPH) (A). Growth of PAK1 KO nmPSCs was significantly less than WT nmPSCs at 72 hours (B). In an orthotopic model where cells of the PAK1-positive murine pancreatic cancer cell line Pan02 were injected into the head of the pancreas of PAK1 KO ($PAK1^{-/-}$) and WT mice, PAK1 expression (ratio to GAPDH) in the tumours from PAK1

KO mice was significantly less than in tumours from WT mice based on staining and protein expression (C). Survival of PAK1 KO mice was significantly greater than for WT mice (D). The data represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to WT.

Figure 5: PAK1 knockdown in pancreatic cancer cells negatively regulated PSCs and the interaction between PSCs and pancreatic cancer cells.

Pancreatic stellate cells incubated in PANC-1 PAK1 knockdown (KD) conditioned media (CM) had reduced PAK1 activity and expression (A) and stellate cell activity (desmin expression) but activation (αSMA expression) was unchanged (B). PANC-1 tumour migration and invasion was significantly increased with increasing ratios of tumour-stellate cells where tumour cell numbers was kept constant and stellate cell numbers were increased (C). In the absence of stellate cells (stellate-tumour cell ratio of 0), tumour cell migration and invasion was reduced in the PANC-1 PAK1 KD tumour clones 2.05 and 2.10 (D). At a stellate-tumour ratio of 50, the migration/invasion of PANC-1 PAK1 KD cells was significantly reduced, but was not significantly different from the values without stellate cells. The data (mean ± SEM) are expressed as a percentage of the corresponding value for the NC1 clone (B), or for the NC1 clone at a stellate-tumour cell ratio of 0 (D), except for pPAK1 expression, which is presented as a percentage of the PAK1 value for the NC1 clone (A). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to negative control or to stellate-tumour cell ratio of 0. ### $p < 0.001$ as indicated in the figure.

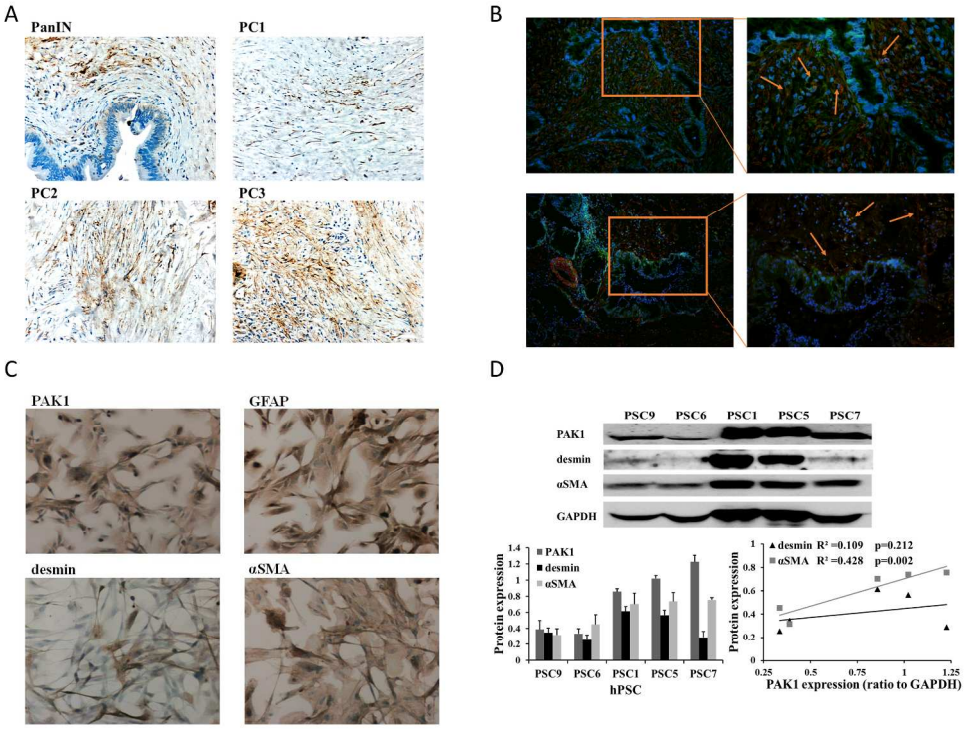

Figure 1: PAK1 is expressed in the stroma of human pancreatic cancer specimens and in isolated pancreatic stellate cells.

Representative images show the varying levels of PAK1 staining in the microenvironment of human pancreatic cancer specimens at x200 magnification (A). Representative images show the co-staining of PAK1 (green) and activated stellate cells (red, staining αSMA) indicated by the orange arrow at x100 (left image) and x200 (right image) magnification (B). Nuclear staining by DAPI is shown in blue and a representative image with the individual colours shown in supplementary figure S2. The purity of pancreatic stellate cells (PSC), isolated from human pancreatic cancer specimens, was confirmed by morphology, and by immunocytochemical staining for desmin, GFAP (glial fibrillary acidic protein) and αSMA (alpha-smooth muscle actin) (C). The human pancreatic stellate cell line hPSC1 and PSC isolated from 4 different human pancreatic cancer specimens expressed varying levels (ratio to GAPDH) of PAK1, desmin and αSMA as detected by western blot (D). PAK1 expression correlated positively with αSMA expression, but not with desmin expression. The data represent mean + SEM, summarised from three independent experiments.

figure 1
254x190mm (300 x 300 DPI)

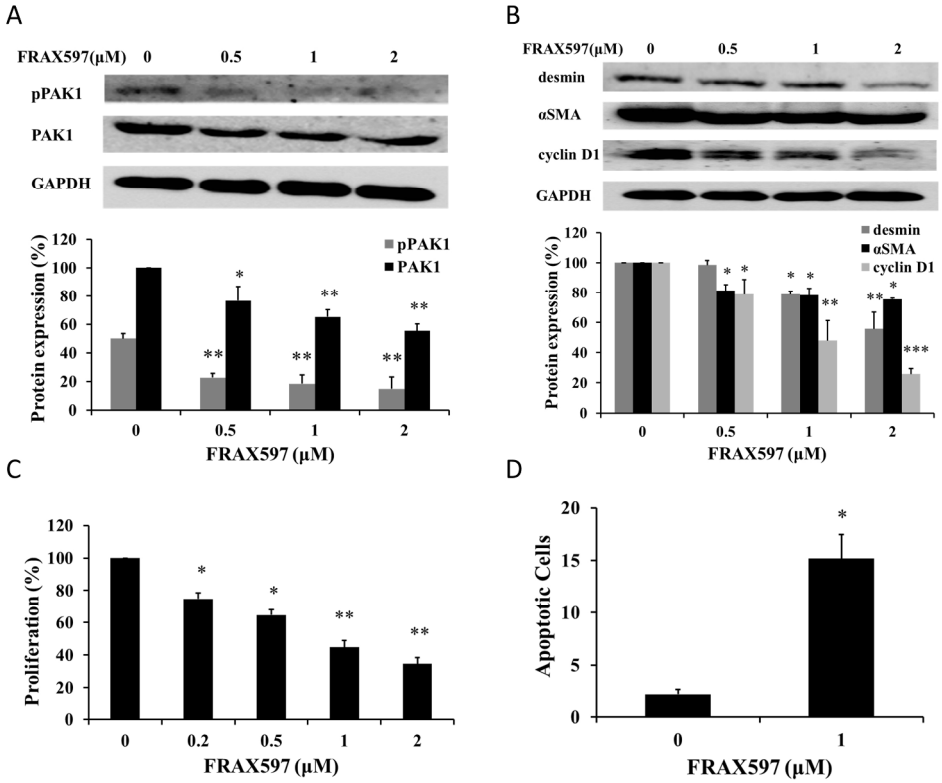

Figure 2: The group 1 PAK inhibitor, FRAX597, decreased PAK1 activity and expression and PSC activation and activity, inhibited PSC proliferation, and increased apoptosis.
FRAX597, a group 1 PAK inhibitor, reduced the activity and expression of PAK1 (A), reduced stellate cell activation (αSMA expression) and desmin expression, and decreased expression of the cell cycle promoter, cyclin D1 (B) after 24 hour treatment on the hPSC1 cell line. FRAX597 inhibited proliferation after 24 hours treatment in a dose dependent manner (C) and increased apoptosis (D). The data (mean + SEM, summarised from three independent experiments) are presented as a percentage to untreated cells, except for pPAK1 expression, which is presented as a percentage of the untreated PAK1 value. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to untreated cells.
figure 2
220x184mm (300 x 300 DPI)

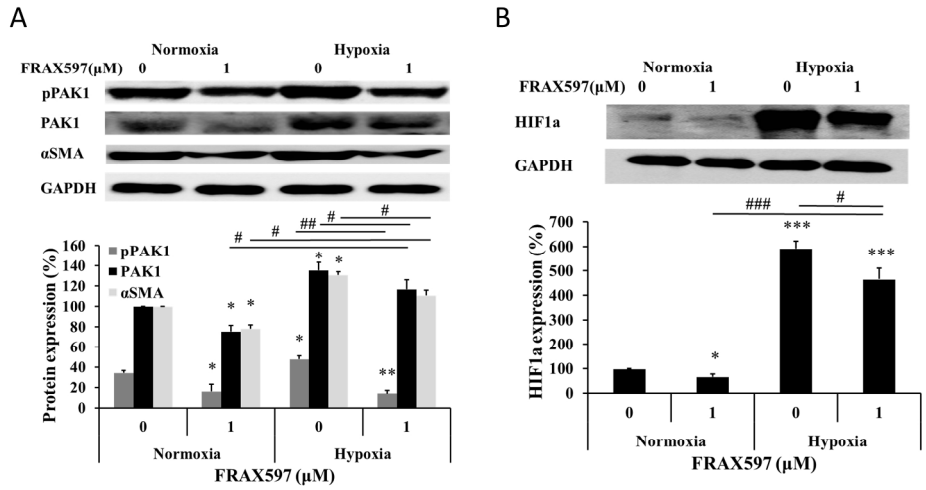

Figure 3: Hypoxia increased PAK1 expression and activity and FRAX597 decreased the expression of αSMA and HIF1α.

PAK1 expression and activity and αSMA expression were increased under hypoxia but were reduced after treatment with 1μM FRAX597 under both normoxia and hypoxia (A) in the hPSC1 cell line. The group 1 PAK inhibitor, FRAX597 also reduced HIF1α expression under both normoxia and hypoxia (D). The data (mean + SEM, summarised from three independent experiments) are presented as a percentage of the corresponding value for untreated normoxic cells, except for pPAK1 expression, which is presented as a percentage of the PAK1 value for untreated normoxic cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to normoxic untreated cells. # $p < 0.05$; ## $p < 0.01$ as indicated in the figure.

figure 3

206x115mm (300 x 300 DPI)

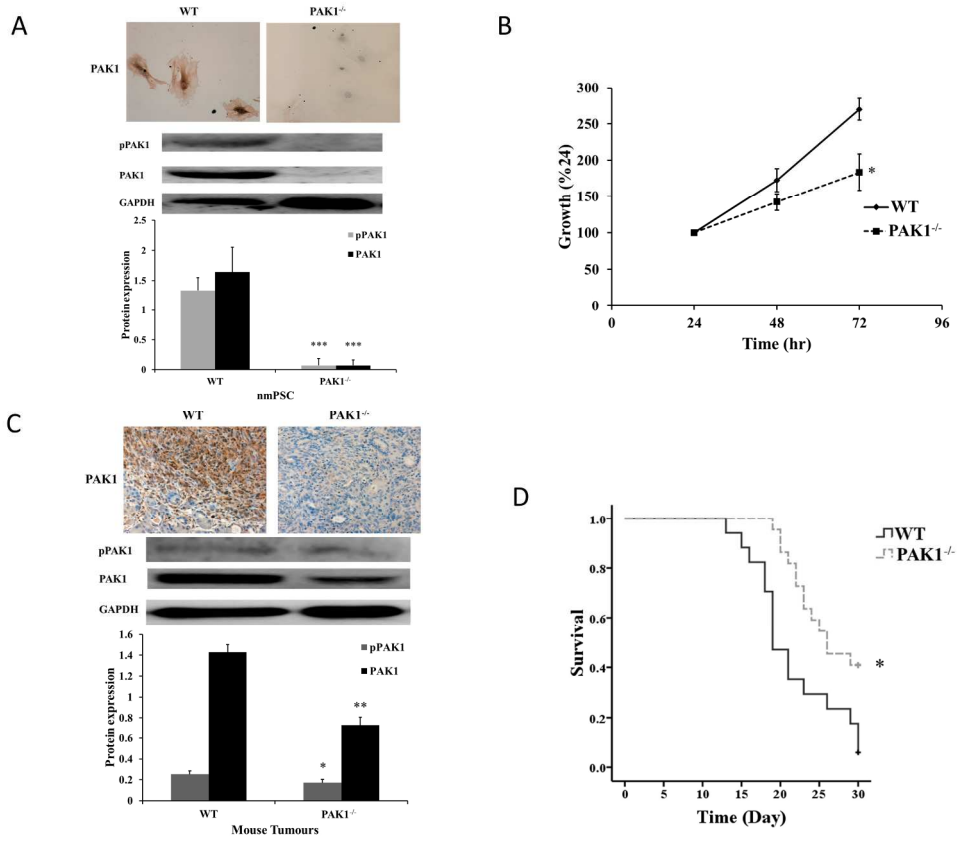

Figure 4: In PAK1 KO mice, growth of quiescent PSCs was reduced, PAK1 expression in pancreatic tumours was decreased, and survival was increased, compared to PAK1 WT mice.
Quiescent murine stellate cells (nmPSC) were successfully isolated from pancreases of PAK1 knockout (KO) mice and shown by immunocytochemistry and western blot (ratio to GADPH) to express negligible amounts of PAK1 (A). Growth of PAK1 KO nmPSCs was significantly less than WT nmPSCs at 72 hours (B). In an orthotopic model where cells of the PAK1-positive murine pancreatic cancer cell line Pan02 were injected into the head of the pancreas of PAK1 KO (PAK1-/-) and WT mice, PAK1 expression (ratio to GAPDH) in the tumours from PAK1 KO mice was significantly less than in tumours from WT mice based on staining and protein expression (C). Survival of PAK1 KO mice was significantly greater than for WT mice (D). The data represent mean + SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to WT.
figure 4
220x189mm (300 x 300 DPI)

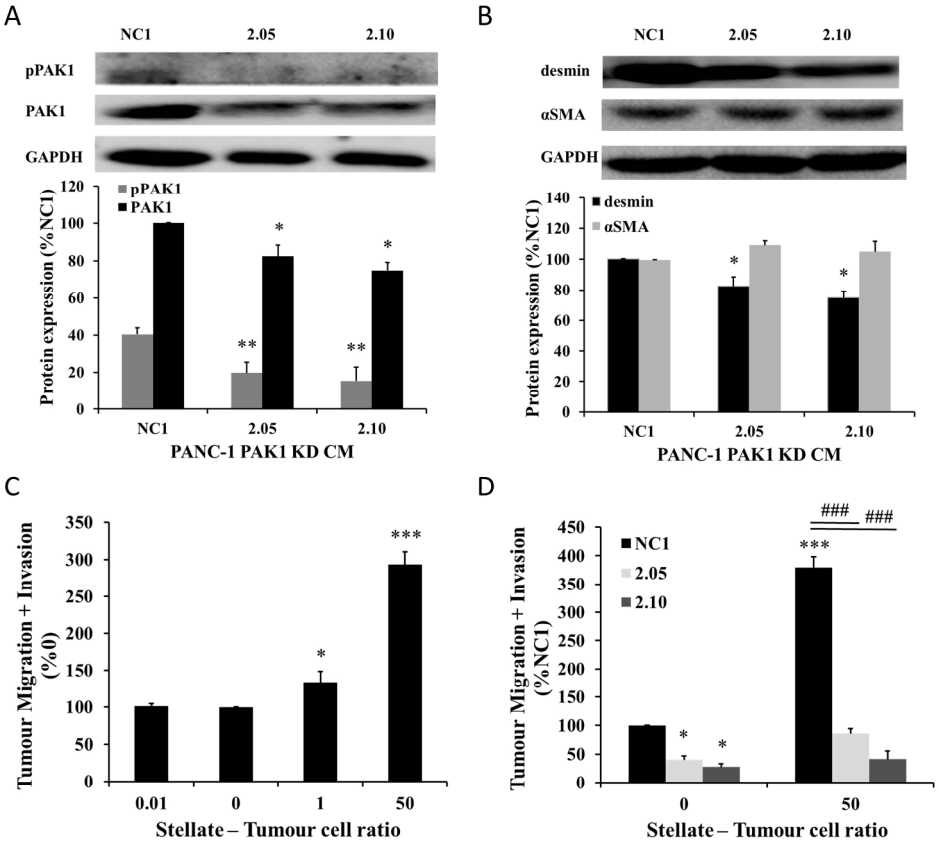

Figure 5: PAK1 knockdown in pancreatic cancer cells negatively regulated PSCs and the interaction between PSCs and pancreatic cancer cells. ‼ ┼ Pancreatic stellate cells incubated in PANC-1 PAK1 knockdown (KD) conditioned media (CM) had reduced PAK1 activity and expression (A) and stellate cell activity (desmin expression) but activation (αSMA expression) was unchanged (B). PANC-1 tumour migration and invasion was significantly increased with increasing ratios of tumour-stellate cells where tumour cell numbers was kept constant and stellate cell numbers were increased (C). In the absence of stellate cells (stellate-tumour cell ratio of 0), tumour cell migration and invasion was reduced in the PANC-1 PAK1 KD tumour clones 2.05 and 2.10 (D). At a stellate-tumour ratio of 50, the migration/invasion of PANC-1 PAK1 KD cells was significantly reduced, but was not significantly different from the values without stellate cells. The data (mean + SEM) are expressed as a percentage of the corresponding value for the NC1 clone (B), or for the NC1 clone at a stellate-tumour cell ratio of 0 (D), except for pPAK1 expression, which is presented as a percentage of the PAK1 value for the NC1 clone (A). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to negative control or to stellate-tumour cell ratio of 0. ### $p < 0.001$ as indicated in the figure.
figure 5
210x185mm (300 x 300 DPI)