Validation of PAK Kinases for the Development of Novel Therapeutics for Pancreatic Cancer Treatment

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ABSTRACT

JASMYN DARDY: Validation of PAK kinases for the Development of Novel Therapeutics for Pancreatic Cancer Treatment (Under the direction of Channing J. Der)

Pancreatic cancer is the 4th leading cause of cancer deaths in the US and essentially all who are diagnosed with this disease will die. Poor patient prognosis is largely due to the lack of effective therapies for the disease. There is only one approved signal transduction targeted therapy that has had marginal clinical value. Thus, there is a dire need for the identification and validation of novel targets that contribute to pancreatic tumorigenesis. Evidence points toward the involvement of PAK kinases as major effectors in cancer. In this study, my overall goal was to determine if I could validate two PAK family kinases, PAK1 or PAK4, as useful therapeutic targets for pancreatic cancer. I found PAK1 and PAK4 protein overexpression in pancreatic cancer tissue and/or cell lines and suppression of PAK expression can impair pancreatic cancer cell growth. My findings support further analysis of PAK as molecular targets and drivers in pancreatic cancer growth.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AKT1</td>
<td>RAC-alpha serine/threonine-protein kinase</td>
</tr>
<tr>
<td>Cdc42</td>
<td>Cell division cycle 42</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanine triphosphatase</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated/extracellular signal-regulated protein kinase</td>
</tr>
<tr>
<td>MTT</td>
<td>3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide</td>
</tr>
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<td>p53</td>
<td>Protein 53</td>
</tr>
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<td>PAK</td>
<td>p21 activated kinase</td>
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<tr>
<td>PBD</td>
<td>p21 binding domain</td>
</tr>
<tr>
<td>PDAC</td>
<td>Pancreatic ductal adenocarcinoma</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology domain</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
<tr>
<td>---------</td>
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<tr>
<td>PIP3</td>
<td>Phosphatidylinositol 3,4,5-triphosphate</td>
</tr>
<tr>
<td>P-Rex</td>
<td>Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor</td>
</tr>
<tr>
<td>Rac1</td>
<td>Ras-related C3 botulinum toxin substrate 1</td>
</tr>
<tr>
<td>Rho</td>
<td>Ras homology family member</td>
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<td>RhoGEF</td>
<td>Ras homology family member guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
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<tr>
<td>Tiam1</td>
<td>T-cell lymphoma invasion and metastasis-inducing protein 1</td>
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1.1 Pancreatic cancer

Pancreatic Cancer is one of the most lethal malignancies in the world and the 4th cause of cancer deaths in the US. Most patients present with advance or metastatic disease that is unresectable, impossible to effectively treat, and ultimately lethal. In 2012, the American Cancer Society estimates that there will be 43,920 new cases, and an estimated 37,390 patients will die from this disease (1). Poor patient survival is largely due to the aggressive nature of the disease, advanced stage upon diagnosis, and the lack of effective therapies for the disease. Despite the advances in our understanding of the molecular biology of the disease, surgical resection remains to be the only curative option, and for the few that qualify, many of these patients experience recurrence after surgery (2, 3). The cytotoxic agent, gemcitabine, provides some benefit for a small fraction of patients, but only modest benefit even for those who do respond, in comparison to its efficacy towards other cancer types. These poor outcomes associated with conventional cytotoxic therapies has driven the cancer field towards the development of molecular targeted therapies, that selectively target the molecular aberrations of the disease that are essential for cancer survival.
To date, the only signal transduction molecularly targeted drug approved for pancreatic cancer treatment is the epidermal growth factor receptor (EGFR) inhibitor erlotinib (4). However, the fact that erlotinib was approved for use, in combination with gemcitabine based on a two week improvement in survival (median 6.24 months in combination with erlotinib versus 5.91 months with gemcitabine alone) emphasizes disappointing state of therapeutic options for pancreatic cancer. Thus, gemcitabine treatment alone remains the standard of care for pancreatic cancer. Because of the limited success with the only approved molecular targeted therapy for the disease, there is a dire need for the identification and validation of novel molecular targets that contribute to pancreatic tumorigenesis. Consequently, a major need and focus of pancreatic cancer research is target discovery and validation. In my studies, I have focused on the PAK1 and PAK4 serine/threonine kinases, with the goal of providing validation for the importance of PAKs as therapeutic targets for pancreatic cancer treatment.

1.2 PAK serine/threonine protein kinases

Evidence points to the involvement of tyrosine kinase and serine/threonine kinase pathways as major effectors in pancreatic cancer development and as potential targets for intervention (5). The p21-activated kinase (PAK) family of serine/threonine kinases are most well characterized as effectors for the Rho GTPases, Rac and Cdc42. Rac and Cdc42 act as molecular switches to regulate processes required during metastasis such as invasion and migration through extracellular matrix, by interacting with PAKs and initiating the kinase cascade.
In pancreatic cancer, Rac may be activated by the K-Ras oncoprotein, that is mutated in essentially all pancreatic ductal adenocarcinomas, which comprise ~80% of all pancreatic cancers. K-Ras may activate Rac through two different effectors, the p110 catalytic subunit of class IA phosphoinositide 3-kinase (PI3K) or the Rac small GTPase guanine nucleotide exchange factor Tiam1. PI3K is a lipid kinase that stimulates phospholipid production, in particular, the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-bisphosphate (PIP3). PIP3 is a membrane-associated lipid that can activate, through binding to pleckstrin homology (PH) domains, the AKT serine/threonine kinase and other proteins. One such PH domain-containing protein is a guanine nucleotide exchange factor (RhoGEF) for Rac, the related P-Rex1 and P-Rex2 isoforms. A second way that K-Ras can activate Rac is through direct binding to another RhoGEF, Tiam1, which selectively activates Rac. That Tiam1 is important for RAS-driven cancer development is shown by experiments where a TIAM1 deficiency reduced the onset of carcinogen (DMBA/TPA)-induced HRAS activation and the development of skin carcinomas (6). That Rac1 is important for KRAS-driven pancreatic cancer development was shown by studies where a deficiency in RAC1, which did not impair normal pancreas growth, did impair pancreatic tumor formation (7). How Rac drives pancreatic cancer growth is currently not known.

PAK kinases are possible candidates for this role.

There are six human PAKs identified thus far, subdivided into two groups: Group I (Pak1-3) and Group II (Pak4-6). PAK1 is the most studied member of Group I and PAK4 is the most studied member of Group II. PAK1 and PAK4 are also both
widely expressed. Both groups have a conserved, N-terminal p21 binding domain (PBD, also called Rho-binding domain: RBD) and a C-terminal serine/threonine kinase domain, but, only Group I possess an autoinhibitory domain (AID), which overlaps with the PBD (Figure 1-1). Rac and Cdc42 are activators of Group I PAKs through binding to the PBD. Consequently, Group II PAKs are essentially constitutively active while Group I PAKs only reach full activation when activated by extracellular signals through GTPase dependent and independent mechanisms, under normal cellular conditions (8). The difference in mode of regulation suggests different cellular functions. PAKs are most notably known for their role in regulating cytoskeletal dynamics and cell motility, but recently they have also been shown to be key regulators of cancer cell signaling networks and function as downstream nodes for various oncogenic signaling pathways, specifically cellular pathways dysregulated in pancreatic cancer.

1.3 PAK and cancer

Research has implicated upregulated expression and activity of PAKs in several human tumor types. PAK1 is the most established family member in human cancers. Initial evidence indicated that exogenously expressed kinase domain-deleted PAK1 inhibited Ras- and Rac- induced transformation of NIH 3T3 mouse fibroblast cells (9). Another study indicated expression of a kinase-dead (K299R) PAK1 blocked K-Ras induced transformation of Rat-1 rat fibroblasts, but surprisingly, not NIH 3T3 cells (10). PAK1 (K299R) is a dominant-negative that inhibits endogenous PAK1 function, presumably by forming inactive homodimer complexes.
Ectopic expression of PAK1 (K299R) reversed the ability of activated H-Ras(G12V) to reduce proliferation and Matrigel invasion and disrupt human immortalized breast MCF-10A epithelial cell ascinar morphology (11). Finally, ectopic expression of PAK1 (K299R) inhibited K-Ras4B growth transformation (soft agar colony formation) of SV40 T Ag-immortalized rat Schwann cells (12). DN PAK1 also reduced the soft agar growth (80%) and tumor growth of NF1-deficient ST88–14 human neuroblastoma cells. Whether PAK1 is required for human tumor cells with endogenously activated mutant Ras has not been determined and is a goal of my studies.

In additional support for a role for PAK2 in cancer, Balasenthil et al was the first group to show increased PAK1 expression in human breast tumors (13). Since then, data from several studies indicate that breast tumor cells use various mechanisms to upregulate PAK1 signaling for survival advantages and perhaps to increase invasive and metastatic potential. Vadlamudi et al was the first to demonstrate a direct link between aberrant PAK1 expression and aggressive breast cancer cell phenotypes by expressing an inducible, constitutively active PAK1 in breast cancer cells (14). The same group also showed in another study that a dominant negative form of PAK1 inhibited breast tumor cell invasiveness (15). Wang et al was the first to report that PAK1 hyperactivation is sufficient to cause mammary-gland tumors in mice suggesting that PAK1 over expression alone is sufficient to induce breast tumorigenesis (16).

Although PAK4 has not been studied as extensively as PAK1, PAK4 is the only family member demonstrated to function as an oncogene. Over expression of
PAK4 also has been observed in many different types of cancer cell lines and tumors including breast, lung, and prostate. PAK4 is chromosomally localized to a region commonly amplified in several human colon and ovarian tumors. Ectopic expression of constitutively active PAK4 leads to anchorage independent NIH 3T3 fibroblast cell growth (17) and kinase dead PAK4 inhibits anchorage independent growth of a human colon cancer cell line. Furthermore, wild-type and constitutively active PAK4 over expression in NIH 3T3 cells stimulated their tumorigenic growth in a nude mouse model, suggesting that PAK4 plays a key role in tumorigenesis (18). This observation with wild type PAK4 is important since mutated PAK4 alleles have not been found in cancer.

Providing further evidence for a role for PAKs in cancer has been the development and evaluation of small molecule PAK inhibitors. In one study a PAK4-selective inhibitor, PF-3758309, showed strong anti-tumor activity when evaluated against a variety cancer types (19). However, no evaluation of any pancreatic cancer cells was done. Furthermore, PF-3758309 is not a very selective PAK4 inhibitor, with activities on other protein kinases, including PAK1. Therefore, it is not entirely clear how important PAK is in pancreatic tumor biology. In another study, a PAK1 selective inhibitor showed potent anti-tumor activity in preclinical cell culture studies (20). This PAK1 inhibitor caused tumor cell apoptosis and was active for both breast and lung cancer cells. No pancreatic cancer cells were tested.
1.4 PAK1 and PAK4 in pancreatic cancer

Although PAK1, PAK4, and other PAK family member have been implicated in multiple cancer types, there have been very limited studies investigating the specific role of PAKs in pancreatic cancer. In a recent study by Billadeau et al, the RhoGEF Vav1, which can activate Rac and Cdc42, contributed to pancreatic cancer tumorigenic properties through the regulation of an EGFR-Vav-Rac-PAK1-cyclinD1 pathway (21). RNAi depletion of Vav1 impaired pancreatic tumor growth in vitro and in vivo. Loss of Vav1 also significantly impaired PAK1 activity, supporting a possible role for PAK1 in Vav1-driven pancreatic cancer growth. Because PAK1 has been shown to play a key role in other cancer types, PAK1 is most likely responsible for the impaired pancreatic cancer cell growth that Billadeau et al attributed to Vav1, although this needs to be tested rigorously. Furthermore, three separate studies identified PAK4 over expression in pancreatic cancer. Amplification of the PAK4 locus has been confirmed in pancreatic cancer (22). Additionally, Kimmelman et al demonstrated a potential role for PAK4 in pancreatic ductal cell motility and invasion (23). I hypothesize that mutant K-Ras-dependent activation of Rac and overexpression of PAK1 and PAK4 can promote pancreatic cancer cell growth. In my studies, I will first determine if PAK1 or PAK4 protein expression is elevated in pancreatic cancer. If overexpressed, then determine if suppression of PAK expression will impair pancreatic tumor growth.
CHAPTER 2.

RESULTS

2.1 PAK protein expression in pancreatic patient tumors and cell lines

Previous studies identified PAK1 overexpression in breast, ovarian and bladder cancer (24). To determine if PAK1 is overexpressed in pancreatic cancer, we used a PAK1 isoform-specific antibody to assess PAK1 protein expression in cell lines and patient tumors. I examined the protein expression of PAK1 in normal, human pancreatic tissue compared to human pancreatic tumor tissue (Figure 2-1).

Consistent with a previous report that none of the PAK isoforms are expressed in normal, human pancreas tissue, two of the three normal tissue samples showed no PAK1 expression (25). Weak expression was observed for one sample. In contrast, most of the tumor samples showed higher PAK1 expression levels when compared to normal tissue. Four of seven tumor samples showed strong PAK1 expression. Two out of seven showed expression similar to the weak expression observed for the only normal sample that exhibited PAK1 expression. Only one of the seven tumor samples showed a weaker expression than the normal sample. These results suggest that PAK1 protein overexpression is found in a subset of pancreatic tumors.

I also examined PAK1 protein expression in a panel of pancreatic tumor cell lines with a PAK1 isoform-specific antibody (Figure 2-2). Consistent with the tumor
analysis results, PAK1 expression was observed in all seventeen cell lines. Several cell lines showed robust PAK1 expression, including the KRAS mutant CFPac-1, Panc-1, and HPAC. Some cell lines showed moderate PAK1 expression. Furthermore, PAK4 expression was also observed in a smaller panel of pancreatic tumor cell lines, using a PAK4 isoform specific antibody (Figure 2-3). Analogous to PAK1 protein expression in PDAC cell lines, a subset of cell lines showed robust PAK4 protein expression and others showed moderate PAK4 expression.

2.2 PAK suppression and PDAC cell anchorage-dependent growth

The aberrant expression of PAK1 and PAK4 prompted my further investigation into the significance of PAK in the in vitro growth properties of pancreatic cancer cells. To address this question, PAK1 and PAK4 suppression in pancreatic cancer cells is required. I used five short hairpin, lentiviral RNA-targeting vectors toward PAK1 in Panc-1 cells to determine which two shRNA vectors gave the best stable suppression of PAK1 protein expression (Figure 2-4). Surprisingly, PAK1sh5 increased PAK1 protein expression compared to Panc-1 cells expressing the non-specific, lentiviral RNA-targeting vector. The PAK1sh4 targeting vector completely depleted PAK1 protein. PAK1sh2 and PAK1sh3 both suppressed PAK1 at comparable levels. Thus, PAK1sh2 and PAK1sh4 were used to suppress PAK1 expression in additional pancreatic cancer cell lines (Figure 2-5). As expected, PAK1sh4 usually gave the better knockdown for each cell line. Weak PAK1 expression was observed for pancreatic cancer cell lines expressing PAK1sh2. To determine which shRNA vectors gave the best stable suppression of PAK4 protein
expression, I used five short hairpin (shRNA), lentiviral RNA-targeting vectors toward PAK4 in Panc-1 cells (Figure 2-6). None of the vectors increased PAK4 protein. The PAK4sh2 targeting vector depleted PAK4 protein the best compared to Panc-1 cells expressing the non-specific, lentiviral RNA-targeting vector. PAK4sh1, PAK4sh4, and PAK4sh5 all suppressed PAK4 at comparable levels. Therefore to be consistent with PAK1 suppression, PAK4sh2 and PAK4sh4 were used to suppress PAK4 expression in additional pancreatic cancer cell lines (Figure 2-7). Both vectors, PAK4sh2 and PAK4sh4 usually gave comparable PAK4 suppression for each cell line.

The anchorage-dependent proliferative potential of pancreatic cancer cell lines depleted of PAK1 and PAK4 was measured by a standard (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT viability assay and cell proliferation assay. Only PAK1sh4 expressing cells showed a significant decrease in proliferation after seven days in the Panc-1 cell line (Figure 2-8). For the MiaPaca-2 and CFPac-1 cell lines, there was no significant difference between the cells expressing the control vector and the cells expressing the PAK1 knockdown vectors (Figure 2-8). Likewise, there was no significant change observed between the cells expressing the control vector and the cells expressing the PAK4 knockdown vectors for the Panc-1, MiaPaca-2, and CFPac-1 cell lines (Figure 2-9). Thus, PAK1 overexpression is essential for the anchorage-dependent growth of one of three cell lines evaluated. On the other hand, PAK4 expression is not essential for the anchorage-dependent growth of all three cell lines tested.
2.3 PAK suppression and PDAC cell anchorage-independent growth

Although PAK1 depletion did not have a robust effect on the proliferative capacity of most of the pancreatic cancer cell lines and PAK4 depletion did not show a significant effect on the proliferative capacity of any of the cell lines tested, PAK depletion may have a greater significance for pancreatic carcinoma cell anchorage-independent growth. Thus, I determined the ability of PAK1-depleted pancreatic tumor cells to form colonies of proliferating cells in soft agar, which is a measure of the ability of cells to proliferate in the absence of attachment. Normal cells lack this ability, whereas tumor cells have escaped the requirement for attachment. The soft agar assay is considered the best in vitro assay that correlates with tumorigenic growth of cells in vivo. I determined that there was a moderate reduction in colony formation for Panc-1 cells expressing PAK1sh2 and a significant reduction in colony formation for Panc-1 cells expressing PAK1sh4, corresponding to the protein level knockdown of each vector (Figure 2-10). PAK1 suppression in MiaPaca-2 cells did not reduce colony formation and some instances resulted in a slight increase (Figure 2-10).

These results parallel to the proliferative capacity of PAK1 inhibited MiaPaca-2 cells observed previously. Surprisingly, PAK1 inhibition in the CFPac-1 cell line showed a robust reduction in colony formation for cells expressing either the PAK1sh2 or the PAK1sh4 vector (Figure 2-10). These results are opposite to the proliferative capacity of PAK1 depleted CFPac-1 cells on plastic. Panc-1 cells expressing the PAK4sh2 vector showed a moderate reduction in colony formation; however, Panc-1 cells expressing the PAK4sh4 vector showed no change (Figure 2-
These results correspond to the protein level knockdown of each vector. Contrastingly, PAK4 suppression in MiaPaca-2 cells showed no change for cells expressing the PAK4sh2 vector (Figure 2-11). A moderate reduction in colony formation was observed for MiaPaca-2 cells expressing the PAK4sh4 vector (Figure 2-11). Similar to PAK1 inhibition in the CFPac-1 cell line, CFPac-1 cells expressing either the PAK4sh2 or the PAK4sh4 vector both showed a moderate reduction in colony formation (Figure 2-11). These different results may reflect distinct roles of PAK1 and PAK4 in different pancreatic cancer cells. Alternatively, there may be off-target activities of the different shRNA vectors used that contribute to the different patterns of growth inhibition. Future studies where I rescue PAK expression with ectopic expression from RNAi-resistant cDNA constructs for PAK1 or PAK4 will be needed to address the specificity of my observations for PAK function in pancreatic cancer.
CHAPTER 3.

DISCUSSION

In my studies, I addressed the question of whether PAK1 and/or PAK4 may be a useful therapeutic target for the treatment of pancreatic cancer. The rationale for my studies is based on the fact that the current standard of care for pancreatic cancer, gemcitabine, is highly ineffective. Pancreatic cancer is the 4th leading cause of cancer deaths in the US and essentially all who are diagnosed with this disease will die. While signal transduction targeted therapies have made important impact on the treatment of other cancers (e.g., imatinib, vemurafenib), the one targeted therapy approved for pancreatic cancer, erlotinib, provided only marginal benefit and is not currently used. My rationale for studying PAK1 is based on three previous findings. First, one study found that Vav1, an activator of the Rac small GTPase, is overexpressed and required for pancreatic cancer tumorigenic growth. They also found that Vav1 activated Rac and its downstream effector, PAK1, although they did not determine if Rac or PAK1 were required for pancreatic cancer growth. Second, two downstream effectors of the K-Ras oncoprotein (PI3K and Tiam1), that is mutationally activated in greater than 90% of pancreatic cancers, can lead to activation of Rac and PAK1. PI3K production of PIP3 can then cause activation of the RacGEF P-Rex1, leading to Rac activation. Tiam1 is a RacGEF that has been shown in mouse models of cancer to be required for RAS-induced skin tumor formation. Third, PAK1 has been shown in cell culture studies to be a positive
regulator of MEK and AKT, key components of Ras effector signaling. Finally, there is evidence for the overexpression and contribution of PAK1 overexpression to other cancers. All these findings provide the rationale for my studies to validate PAK1 in pancreatic cancer.

In this study, I provide evidence to support further analyses investigating the significance of PAK1 and potentially PAK4 as drivers in pancreatic cancer growth and PAK1 and PAK4 inhibitors as a novel therapeutic approach for the treatment of this deadly disease. My analyses of human tissue samples provide strong preliminary evidence that PAK1 protein has a higher incidence of aberrant overexpression in tumor versus normal tissue. These findings are surprising because a study by Arias-Romero et al revealed no transcriptional expression of all six PAK family members in normal pancreas tissue based on SAGE (serial analysis of gene expression), and suggests that the high levels of PAK1 protein that I found is aberrant and tumor-specific (25). Moreover, SAGE revealed widespread distribution of the PAK family members in almost all normal human tissues examined with the exception of the pancreatic tissue. My studies only evaluated PAK1 protein expression in a limited number of patient tumors. Consequently, a more comprehensive evaluation of a larger number of tumors are needed to determine if PAK1 protein expression and other PAK isoforms are associated with specific genetic mutations in pancreatic cancer, which may give me an idea of how overexpression may be occurring. Also, a comparison of primary and metastatic pancreatic tumors may determine if overexpression occurs during primary tumor formation or if it is associated with progression to metastatic cancer.
The aberrant PAK protein expression observed in the PDAC cell lines not only coincided with the tumor analysis results, but also further validated the importance of PAK1 as a therapeutic target for pancreatic cancer treatment. In fact, a recent study revealed that high PAK1 protein levels may predict tamoxifen insensitivity (26). Together, this study and the PAK1 protein overexpression observed in both the PDAC cell lines and patient tumors raise the question: Does the aberrant expression of PAK1 promote pancreatic cancer resistance to chemotherapeutic agents? An important future study will be to determine if PAK1 inhibition will enhance the sensitivity of pancreatic tumor cells to gemcitabine.

My study furthermore provides strong preliminary evidence regarding the significance of PAK in the in vitro growth properties of pancreatic cancer cells. Further studies of the consequences of PAK knockdown, specifically PAK1 and PAK4, on the growth of pancreatic cancer cell lines in vivo now need to be done. This could involve the standard nude mouse subcutaneous tumor xenograft studies. Even better would be the use of orthotopic tumor models where pancreatic tumor cells are inoculated into the pancreas. In these models, pancreatic cancers cells can invade and metastasize to organs that are seen in the cancer patient. This model would then determine if PAK is important for initial primary tumor growth as well as the invasive and metastatic properties. Another approach for in vivo validation of PAK would be to perform a conditional loss of PAK in pancreatic tissue in the widely used mutant KRAS/p53 mouse model of pancreatic cancer formation. In this model, if PAK is lost concurrently with KRAS activation, it would then determine if PAK is required for tumor progression. A more relevant study for cancer
treatment will be to use inducible shRNA for PAK1 to then silence PAK1 expression in already developed pancreatic tumors.

Another important future study to validate PAK can be the study of a pharmacologic inhibitor of PAK to test in mouse models of pancreatic cancer. Recently, a PAK1 inhibitor was described and shown to have anti-tumor activity. I could use this inhibitor to treat preformed human xenograft tumors or the mouse tumors that develop in the KRAS/p53 mouse model. I would then determine if tumor progression is reduced to increase mouse survival or if tumor regression is seen. If tumor regression is seen, then I can determine if this is occurring because the loss of PAK1 is needed for cell survival and blocking PAK1 is causing apoptosis.

Although my evaluation of PAK1 and PAK4 in pancreatic cancer cell lines was limited, some conclusions regarding PAKs potential as therapeutic targets can be inferred and these inferences prompt further investigation. Because I found that PAK1 overexpression is important for the anchorage-dependent growth of only one of three cell lines evaluated, this suggests that there may be a subset of pancreatic cancer cells that are dependent on PAK1 for anchorage-dependent growth and a subset that are not dependent. These findings are not completely shocking, as a recent study classified cancer cell lines into K-Ras dependent and K-Ras independent groups (27). Furthermore, I only used shRNA suppression of PAK1 and, RNAi suppression is not a complete knock out of the gene. Therefore, residual PAK1 protein may be sufficient in certain cell lines to support PAK activity. In addition, rescue studies must also be completed to confirm that there are no off-target activities of the shRNA vectors. Although it is unlikely, other PAK isoforms
could potentially compensate for PAK1 and suppression. PAK4 overexpression was not important for the anchorage-dependent growth of the cell lines tested, which is not completely surprising because PAK4 has been more closely linked to metastatic and invasive properties of cells. Likewise, the data regarding anchorage-independent growth suggests that there may also be a subset of pancreatic cancer cells that are dependent on PAK1 for anchorage-independent growth and a subset that are not dependent. Again, additional studies are required to deduct residual PAK activities, off-target activities, and other PAK isoform compensation. Nevertheless, these findings support further analysis using a larger panel of PDAC cell lines.

In summary, I believe that my preliminary studies provide evidence to continue the study of PAK1 and PAK4 in pancreatic cancer. Because PAKs are protein kinases, they are much more of druggable targets than mutant K-Ras. So, if further studies provide strong evidence for the role of PAK1 and/or PAK4 in pancreatic cancer, PAK1 and/or other PAK isoforms may finally give us the anti-Ras drug that has been searched for in the last 30 years.
CHAPTER 4.
METHODS

Antibodies, Primary Tumors, and Cell Culture

Polyclonal antibodies for PAK1 (cat. No, vendor) and PAK4 (cat. No, vendor), specifically, were obtained from Cell Signaling Technology Inc. Monoclonal GAPDH antibody was obtained from BD Bioscience.

Normal pancreas and tumor tissue were provide by Jen Jen Yeh, M.D, at UNC School of Medicine. All cell lines (Panc-1, MiaPaca-2, and CFPac-1) were reported to be derived from human pancreatic duct carcinoma and obtained from the American Type Culture Collection. Panc-1 and MiaPaca-2 cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum. CFPac-1 cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum. All cell lines were grown under standard culture conditions (5% CO₂, 95% air in humidified chamber at 37°C).

Anchorage-dependent proliferation assay

To monitor the contribution of PAK to tumor cell anchorage-dependent growth, confluent cell cultures were trypsinized and seeded into 96-well plates in concentrations of 3x10³ cells/well. After 1, 3, 5, and 7 days, cells were stained with 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and
incubated at 37°C for 4 h. Fifty µl of vehicle (DMSO) was added to each well after aspirating MTT. The absorbance of the solution was measure using a spectrophotometer. The average number of cells on duplicate wells was calculated.

**Soft agar colony formation assay**

Confluent cell cultures were trypsinized and seeded into 6-well plates in concentrations of 5x10³ cells/well as single cell suspensions in 0.3% bacto-agar in complete growth medium. The single cell suspensions were layered on top of 0.6% bacto-agar in complete growth medium. After 2-4 weeks, colonies were stained with 2 mg/ml MTT and the average number of colonies on duplicate dishes was calculated using ImageJ.
CHAPTER 5.  
FIGURES

**Figure 1.1. PAK protein structure.** PAKs (p21-activated kinases) were first identified in screens for Rac and Cdc42 effectors and independently as a proteinase-activated kinase [1, 2]. There are six human PAK isoforms subdivided into Group I (PAK1-3) or Group II (PAK4-6). All PAKs are characterized by an N-terminal regulatory domain and a highly conserved C-terminal kinase domain. Overall sequence identity is indicated at the left and kinase domain sequence identity within each Group is indicated below the kinase domain. The regulatory domains of all PAKs consist of Rho GTPase-binding domain (RBD) and several proline-rich regions that serve as docking sites for SH3 domain-containing proteins. Group I Paks additionally possess an autoinhibitory domain (AID) overlapping with the RBD and a binding site for the PIX guanine nucleotide exchange factors.
Figure 2.1. PAK1 protein expression in pancreatic tissue. Normal or pancreatic tumor tissues were lysed and resolved by SDS-PAGE. Blot analyses were done with anti-PAK1 antibody and anti-GAPDH antibody to verify equivalent loading of total protein.
Figure 2.2. PAK1 protein expression in PDAC cell lines. The indicated cell lines were lysed and resolved by SDS-PAGE. Blot analyses were done with anti-PAK1 antibody and anti-GAPDH antibody to verify equivalent loading of total cellular protein.
Figure 2.3. PAK4 protein expression in PDAC cell lines. The indicated cell lines were lysed and resolved by SDS-PAGE. Western blot analyses were done with anti-PAK4 antibody and anti-GAPDH antibody to verify equivalent loading of total cellular protein.
Figure 2.4. Evaluation of lentivirus vectors expressing shRNA targeting human PAK1. Lentivirus-based shRNA vectors targeting different sequences of human PAK1 were obtained from the UNC vector core and were stably-infected into Panc-1 cells, followed by puromycin selection, with multiple drug-resistant colonies pooled together. Blot analyses were done with anti-PAK1 antibody and anti-GAPDH antibody to verify equivalent loading of total cellular protein. The sh2 and sh4 shRNA vectors showed the most consistent and reproducible suppression of PAK1 protein expression and were utilized for all subsequent studies.
Figure 2.5. PAK1 suppression in pancreatic cancer cells. Lentivirus-shRNA vectors sh2 or sh4 were stably-infected into the indicated pancreatic carcinoma cell line followed by puromycin selection, with multiple drug-resistant colonies pooled together. Blot analyses were done with anti-PAK1 antibody and anti-GAPDH antibody to verify equivalent loading of total cellular protein.
Figure 2.6. Evaluation of lentivirus vectors expressing shRNA targeting human PAK1. Lentivirus-based shRNA vectors targeting different sequences of human PAK4 were obtained from the UNC vector core and were stably-infected into Panc-1 cells followed by puromycin selection, with multiple drug-resistant colonies pooled together. Blot analyses were done with anti-PAK1 antibody and anti-GAPDH antibody to verify equivalent loading of total cellular protein. The sh2 and sh4 shRNA vectors showed the most consistent and reproducible suppression of PAK4 protein expression and were utilized for all subsequent studies.
Figure 2.7. PAK4 suppression in pancreatic cancer cells. Lentivirus-shRNA vectors sh2 or sh4 were stably-infected into the indicated pancreatic carcinoma cell line, followed by puromycin selection, with multiple drug-resistant colonies pooled together. Blot analyses were done with anti-PAK4 antibody and anti-GAPDH antibody to verify equivalent loading of total cellular protein.
Figure 2.8. PAK1 suppression does not impair anchorage-dependent proliferative. The proliferation of the indicated pancreatic cancer cell lines depleted of PAK1 was measured by a standard MTT viability assay. Data shown are representative of at least three independent experiments.
Figure 2.9. PAK1 suppression does not impair anchorage-dependent proliferative. The proliferation of the indicated pancreatic cancer cell lines depleted of PAK4 was measured by a standard MTT viability assay. Data shown are representative of at least three independent experiments.
**Figure 2.10.** PAK1 suppression impairs anchorage-independent proliferation of some PDAC cell lines. The colony formation in soft agar of the indicated pancreatic cancer cell lines depleted of PAK1 was quantitated approximately 14 days. Data shown are representative of at least three independent experiments. Error bars are the mean ± standard deviation for two duplicate dishes.
Figure 2.11. PAK4 suppression impairs anchorage-independent proliferation of some PDAC cell lines. The colony formation in soft agar of the indicated pancreatic cancer cell lines depleted of PAK4 was quantitated after approximately 14 days. Data shown are representative of at least three independent experiments. Error bars shown are the mean ± standard deviation for duplicate dishes.
REFERENCES


