A NOVEL ROLE FOR SMAD6 IN THE BIOLOGY OF PANCREATIC CANCER

by
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ABSTRACT

Pancreatic cancer is a nearly uniformly lethal disease, with the five-year survival rate remaining a dismal five percent. Recently, our laboratory has associated the loss of SMAD4, a critical mediator of TGF-β and BMP signaling, with the presence of widely disseminated pancreatic cancer. Therefore, we sought to obtain a better understanding of these pathways in the biology of pancreatic cancer through analysis of SMAD4 loss and SMAD6 elevation.

To gain a better understanding of the role of SMAD4 loss in pancreatic cancer, we established isogenic cell lines from pancreatic cancer cells that were SMAD4-deficient, such that these cell lines were SMAD4-complemented. We were able to show that restoration of SMAD4 into these cell lines hindered their ability to proliferate, migrate, and invade through matrigel *in vitro*. Furthermore, we sought to gain an understanding of the utility of SMAD4 as a marker for the efficacy of chemotherapy. We have shown that SMAD4 status is predictive of a small, but statistically significant, *in vitro* response to cisplatin and irinotecan and resistance to gemcitabine.

Second, we identified SMAD6, previously described as an inhibitor of both TGF-β and BMP, as being differentially expressed in pancreatic cancer cell lines and tissues. SMAD6 expression was found not to be regulated by genetic mutation or degree of RNA transcription, but through aberrant proteolytic degradation. It is through loss of this regulatory mechanism that SMAD6 expression becomes elevated, leading to increased levels of proliferation, migration, and invasion through matrigel, *in vitro*, and increased metastatic burden in patients. Despite its previously described role as an inhibitor of

TGF-β and BMP signaling, we have determined that the effects of SMAD6 on pancreatic cancer cells are independent of this function. Rather, we have identified a nuclear role for SMAD6: SMAD6 is able to bind to DNA in pancreatic cancer, and is constitutively located in the nucleus of pancreatic cancer cells.

Therefore, both SMAD4 loss and SMAD6 overexpression negatively impact the biology of pancreatic cancer, albeit through different mechanisms. Further study will continue to elucidate the role of these proteins in the biology of pancreatic cancer.

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CHAPTER 1. INTRODUCTION

Pancreatic Cancer

Pancreatic ductal adenocarcinoma (hereafter referred to as pancreatic cancer) is an almost uniformly lethal disease – it was estimated that eighty-five perfect of patients diagnosed with pancreatic cancer in 2012 succumbed to their disease (1). Its five-year survival rate of merely five percent has remained constant over many years; as such, pancreatic cancer is the fourth-leading cause of cancer-related death in the United States (1,2).

The dire statistics attributed to pancreatic cancer are due, largely, to two major factors. First, there currently are no effective and established screening modalities for pancreatic cancer (3). Surgery, the only potentially curative treatment for pancreatic cancer, is not an option for up to eighty percent of patients due to the advanced stage at which their diagnosis occurred (4-6). As such, there has been fervent interest in developing techniques to catch pancreatic cancer at an early stage. Recent studies suggest progress in the discovery of pancreatic cancer biomarkers. A panel of microRNAs, in combination with CA19-9, has shown preliminary effectiveness in distinguishing the blood of patients with pancreatic cancer from that of healthy individuals (7). In addition, methylation of two particular genes, *BNC1* and *ADAMTS1*, in serum has shown promise in the detection of early stage pancreatic cancer (8).

Aside from the hurdle of detecting pancreatic cancer while the disease is in a manageable state, effective non-surgical means for treating the disease remain limited. Gemcitabine, the chemotherapeutic standard of care for managing pancreatic cancer, is not particularly effective on its own (4,6,9). Recent studies have indicated that gemcitabine is more effective when it is used in combination with other agents, including

erlotonib, Nab-paclitaxel, capecitabine, and docetaxel (10-12). In addition, recent success has been reported using a combination of oxaliplatin, irinotecan, fluorouracil, and leucovorin (FOLFIRINOX) (13). Furthermore, most patients who undergo surgical resection of their pancreatic cancer eventually have disease recurrence (14). As such, there is significant interest in the development of improved adjuvant treatment regimens – including chemotherapy and radiation strategies – to lower this probability (14,15).

Pancreatic Cancer Progression and Genetics

Pancreatic cancer develops through several known precursor lesions, including mucinous cystic neoplasms (MCNs), intraductal papillary mucinous neoplasms (IPMNs), and pancreatic intraepithelial neoplasias (PanINs), with the latter being the most common of the three (16). PanINs form in the smallest pancreatic ducts and are characterized by increasing morphological changes from PanIN1 to PanIN3 lesions (17-19). PanIN lesions have also been characterized genetically; the alterations that are known to occur at each stage are discussed below.

Both targeted and global sequencing studies in pancreatic cancer have revealed four genes that are mutated at particularly high frequency in pancreatic cancer. The predominant mutation that is known to drive pancreatic cancer progression occurs in *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) in codons 12 and 13 (20-22). Mutations in this gene occur in up to 99% of pancreatic cancers (21,22). *KRAS* encodes a GTPase known to activate many downstream pathways, including mitogen-activated protein kinase and phosphoinositol-3 kinase networks (reviewed in (23)). Of the four major driver mutations in pancreatic cancer, *KRAS* is the only gain-of-function

mutation. The next most common genetic event in pancreatic cancer is the inactivation of the cyclin-dependent kinase inhibitor *CDKN2A/p16* (cyclin-dependent kinase inhibitor 2A), through mutation along with allelic loss, homozygous deletion, or promoter hypermethylation (24-26). Mutations in *KRAS* and *CDKN2A* are found in early PanIN lesions (PanIN1-2), with *KRAS* believed to be the first mutation of the two to occur (27).

Conversely, mutations in the other driver genes in pancreatic cancers tend to occur as late as PanIN3 (28,29). Loss of *TP53* (tumor protein 53) function occurs through point mutation or small intragenic deletion in three-quarters of pancreatic cancers (30,31). This protein is a tumor suppressor involved in many cellular functions (reviewed in (32)). *SMAD4/DPC4* is the final common alteration in pancreatic cancer, with mutations or homozygous deletions occurring in 55% of cases (33). Mutations in *SMAD4* cause the protein to be rapidly degraded (34). Therefore, immunohistochemistry for the presence or absence of SMAD4 is commonly used to assess SMAD4 status in pancreatic cancer patients (35). SMAD4 is a transcription factor necessary for TGF-β and BMP signaling (36,37). It is worth noting that additional members of these two pathways have been found to harbor mutations at low frequencies in pancreatic cancer (21,22,38,39).

Interestingly, the mutations that occur in the aforementioned genes are not random. Loss of *SMAD4* tends to occur in cancers that also harbor *TP53* alterations, suggesting that *TP53* loss precedes that of *SMAD4* (40). Furthermore, the types of mutations observed in these genes are closely linked to each other. *SMAD4* loss tends to occur in association with *TP53* missense mutations, whereas cancers with wild-type *SMAD4* are more likely to harbor nonsense or frame-shift mutations or homozygous

deletions of *TP53* (40). Further research is needed to parse out the true meaning behind these genetic patterns.

Pancreatic Cancer Metastasis

While the genetic events that are associated with early-stage pancreatic cancer have been well-characterized, the alterations leading to the metastatic dissemination of pancreatic cancer are not well understood. As such, our laboratory established the Gastrointestinal Cancer Rapid Medical Donation Program (GICRMDP) at Johns Hopkins in 2003 in order to better study the events that underlie end-stage pancreatic cancer (41). As part of this IRB-approved program, rapid autopsies are performed, on average, eight hours post-mortum on consenting gastrointestinal cancer patients, including those that have suffered from pancreatic cancer. As part of the autopsy, primary tumor tissue, metastatic lesions, and normal samples are collected and saved for downstream analysis. Several important observations regarding pancreatic cancer metastasis have arisen from the research associated with this program. First, there are two distinct patterns of metastasis in pancreatic cancer patients: approximately two-thirds of patients display widespread metastasis at autopsy, while the remaining patients have a more localized disease (42). Of the patients with widespread disease, the most common sites of metastasis are the liver, peritoneum, and lung, although the disease will rarely spread to bone, the adrenal glands, as well as other sites.

In addition, the genetics of pancreatic cancer metastasis have become clearer through the study of pancreatic cancer autopsy samples. Alterations of *TP53* and *SMAD4* were both independently associated with widespread metastasis (42). Furthermore,

Yachida and colleagues, through whole-exome sequencing of seven patients' primary and metastatic lesions, were able to better elucidate the process and timing of pancreatic cancer metastasis (43). Through a comparison of the mutations in the metastases and their corresponding primary cancers, a genetic progression model could be established for each patient. Comparative lesion sequencing revealed that genetically discrete subclones exist within the primary tumor, leading to the formation of distinct distant metastases (43,44). Furthermore, based on a calculated proliferative rate and the number of mutations found in each lesion, a timeline of pancreatic cancer formation was able to be gleaned. The development of the parental clone of the tumor takes nearly twelve years, the formation of metastatic subclones takes an additional seven years, and the formation of frank, distant metastases takes an additional three years. All in all, the time from pancreatic cancer initiation to death is approximately twenty-one years, leaving a large window of opportunity for clinical intervention (43).

Transforming Growth Factor-beta (TGF-β) and Bone Morphogenetic Protein (BMP) Signaling

As previously stated, SMAD4, one of the major driver genes of pancreatic cancer, is a central mediator of TGF-β and BMP signaling (36). These pathways, which comprise the TGF-β superfamily of signaling, are summarized in Figure 1. Global sequencing analyses have confirmed these pathways to be among the most highly dysregulated in pancreatic cancer (21,22). The TGF-β superfamily can be divided into

the TGF-β and BMP tracts, each of which relay their signal through similar kinase cascades.

TGF-β ligands bind to TGF-β receptor II (TGF-βRII), which is a constitutively active serine-threonine kinase (45,46). This event stimulates the formation of a complex between TGF-βRII and TGF-β receptor I (TGF-βRI); upon complex formation, TGF-βRII phosphorylates TGF-βRI, activating the latter, which is also a serine-threonine kinase (45,47). Upon presentation to TGF-βRI by the Smad anchor for receptor activation (SARA), the substrates of TGF-βRI, SMAD2 and SMAD3 (referred to as R-SMADs) are phosphorylated (47-50). These effectors then dissociate from SARA and bind to SMAD4 in the cytoplasm (33,36,47,49,50). This complex then enters the nucleus and is able to bind to SMAD-binding elements in the promoter regions of DNA in conjunction with other transcription factors (37,51,52).

The BMP pathway proceeds in a similar manner. BMP ligands bind to type II receptors in concert with type I receptors, much like their TGF-β counterparts (53-56) The BMP-specific R-SMADs, SMAD1, SMAD5, and SMAD8, are subsequently phosphorylated after presentation to the receptor by ENDOFIN (57-61). Upon association with SMAD4, the R-SMAD heteromeric complexes enter the nucleus to affect transcription (36,57,60).

It should be noted that TGF-β and BMP signaling are both tightly regulated within cells. A large number of endogenous antagonists have been identified for both pathways, acting at several points along the pathways under several different mechanisms, including mimicking receptors, competitively binding pathway components, enhancing degradation of pathway components, and converting the SMAD-containing

transcription activating complex to a repressive complex (62-85). These inhibitors, along with specific functions of each, are listed in Table 1.

TGF-β and BMP Signaling in Pancreatic Cancer

SMAD4 was originally identified based on its frequent deletion in pancreatic cancer (its original name is Deleted in Pancreatic Cancer, Locus 4) (33). Further research has indicated that SMAD4 mediates suppression of cell proliferation through the activation of the cell cycle inhibitors *p21/waf1* and *p15/ink4b* (86-88). In addition, the modulation of SMAD4 levels in pancreatic cancer cells has caused a reduction in growth rate and angiogenesis using a xenograft model (89-91). Furthermore, SMAD4 has been shown to reduce the degree to which pancreatic cancer cells are able to migrate and invade in response to TGF-β *in vitro*, with the latter potentially affected by a decrease in MMP-2 and MMP-9 production (90,92). Gene expression studies confirm that SMAD4 loss causes changes in the gene expression patterns of several relevant pathways: cytoskeleton remodeling, cell cycle regulation, and proliferation (86). Thus, the perturbation of TGF-β and BMP signaling through SMAD4 loss affects pancreatic cancer cells in numerous ways that could contribute to the progression of pancreatic cancer. Furthermore, multiple studies have implicated SMAD4 loss with a worse prognosis in pancreatic cancer (93,94).

These observations suggest that loss of TGF-β and BMP signaling through *SMAD4* mutation or deletion is an oncogenic event in pancreatic cancer. However, more broad modulation of these pathways has yielded conflicting information. Supplementing

pancreatic cell media with TGF-β promoted epithelial-to-mesenchymal transition and enhanced the production and activity of the pro-metastatic factors matrix-metalloproteinase 2, urokinase plasminogen activator, and urokinase plasminogen activator receptor (95-98). Pharmacological inhibition of TGF-β receptors inhibited the migration and invasion of COLO-357 and BxPC3, but not Panc1, pancreatic cancer cells (99,100). Similarly, mice harboring *KRAS* and *TP53* mutations in their pancreata displayed accelerated PanIN and pancreatic cancer formation when treated with a TGF-β neutralizing antibody (101). Furthermore, the mice in which TGF-β was blocked had more rapidly proliferating tumors and reduced surivival compared to control mice. These results confirm those of Rowland-Goldsmith *et al.*, who showed that inhibiting TGF-β signaling in a xenograft model of pancreatic cancer blocks tumor growth, angiogenesis, and metastasis (102). In patients, mutation of *TGF-βRII* does not affect survival, while an elevated presence of TGF-β ligands confers a poor prognosis (93,103).

BMP modulation also yields conflicting results. The treatment of pancreatic cancer cells with BMP-2 has a SMAD-4 dependent effect on proliferation rate – BMP-2 increased the proliferation of the SMAD4-deficient cell lines, AsPC -1 and Capan1, decreased the proliferation of the SMAD4-intact cell line, COLO-357, and had no effect on the SMAD4-intact cell lines MiaPaCa2, Panc1, and T3M4 (104). These changes in cell proliferation were rescued by the complementation (AsPC-1 and Capan1) or inhibition (COLO-357) of SMAD4 in these cell lines. Most of what is known regarding the effect of BMP on cell motility has been shown in the Panc1 cell line. Treatment of Panc1 with BMP-2, -4, or -7 stimulated an epithelial-mesechymal transition (105-107). Furthermore, treatment of Panc1 with these BMP ligands stimulated invasion and

production of MMP-2 (106). However, loss of BMPR1A has been shown to negatively impact patient survival, more closely mirroring loss of SMAD4 (93,94,108).

SMAD6

One inhibitor of TGF-β and BMP signaling, SMAD6, has been of particular interest since its cloning in 1997 (74,109). This inhibitory SMAD was originally identified to bind TGF-β and BMP type I receptors in a ligand-dependent manner, subsequently inhibiting the phosphorylation of SMAD1 and SMAD2, but not SMAD3 (74). As such, SMAD6 inhibited the activity of the TGF-β-specific luciferase reporter, p3TP-Lux (74,109). However, additional studies called into question the ability of SMAD6 to inhibit TGF-β signaling. Hata *et al.* determined that SMAD6 expression inhibited the transcription of SMAD1 target genes, but not those of SMAD2 (75). Furthermore, this group did not observe a change in p3TP-Lux activity, while a BMP-specific luciferase reporter showed a significant reduction (75). While some refer to SMAD6 as being BMP-specific in its actions, it has been shown to affect TGF-β signaling as well (110,111).

The mechanisms through which SMAD6 has been proposed to inhibit signaling through the TGF-β superfamily are varied. SMAD6 interacts with BMPR1 and TGF-βRI *in vitro* (74,75,77). It is thought that SMAD6 recruits the ubiquitin ligase, SMURF1, to the type 1 receptors, facilitating their degradation (77). Furthermore, SMAD6 was shown to recruit SMURF1 to the BMP-specific R-SMADs, SMAD1 and SMAD5, targeting

them for degradation, as well. In addition, SMAD6 was able to bind to SMAD1, inhibiting its binding to SMAD4 (75).

In addition to these cytoplasmic mechanisms for SMAD6 function, there exists a nuclear component, as well. SMAD6 was found to bind to several transcriptional co-repressors, including HOXC8, HDAC1, and CTBP (76,112,113). When in complex with these factors, SMAD6 is able to act as part of the repressive complex through its MH1 domain (112). Target genes for SMAD6-mediated repression include *ID1, ID2, Osteopontin,* and *HHEX* (76,113,114). SMAD6 has also been shown to interrupt the DNA-binding ability of additional transcription factors, including DLX3 and TBX6 (115,116). Additional "non-canonical" functions of SMAD6 include inhibition of the Wnt and NFκB signaling (117,118)

Of particular interest to us, SMAD6 has been previously associated with pancreatic disease. Mice overexpressing this protein in their pancreatic acinar cells were more susceptible to developing pancreatitis, which is considered a risk factor for pancreatic cancer (119,120). In pancreatic cancer, *SMAD6* has been shown to have elevated expression at the RNA level (110). Overexpression of SMAD6 in COLO-357 pancreatic cancer cells increased anchorage independent growth, while suppressing TGF-β mediated growth inhibition. In other cancer models, SMAD6 increases cell growth and clonogenicity of non-small-cell lung cancer cells, while reducing survival (111). Furthermore, a SNP in SMAD6 is associated with increased probability of brain metastasis in non-small-cell lung cancer (121).

Given our interest in the metastasis of pancreatic cancer and our prior association of SMAD4 loss with widely metastatic disease (42), we sought to better characterize the effects of TGF-β and BMP pathway loss on the biology of pancreatic cancer. Herein, we describe the effects of the modulation of SMAD4 and SMAD6 on pancreatic cancer cells.

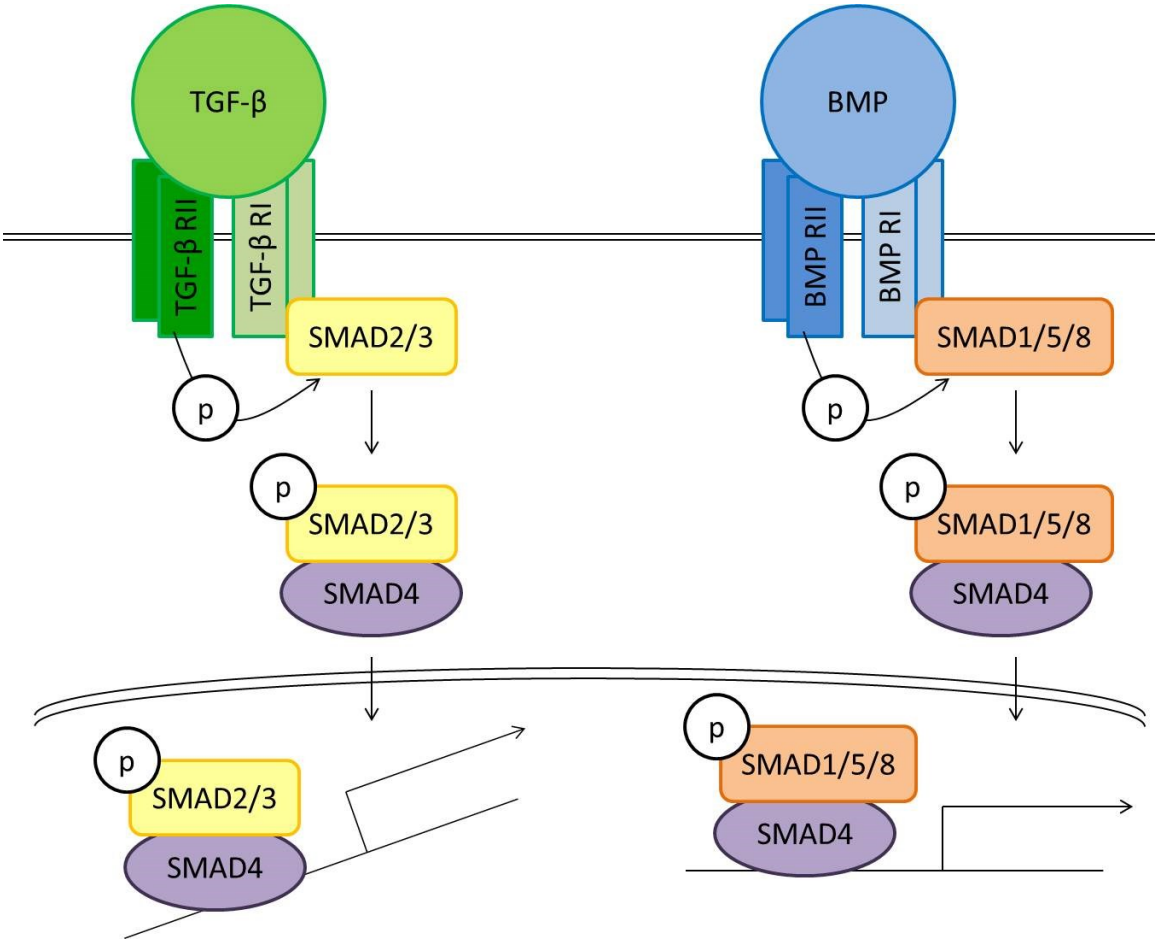

Figure 1. Schematic of the TGF-β and BMP pathways.

Table 1. Functions of endogenous TGF-β and BMP inhibitors.

Protein	Function	Reference
PIN1	Induces degradation of SMAD2/3 by recruitment of SMURF2	(62)
SMURF1	Induces ubiquitination of SMAD1/5/8, TGF-βRI	(63,64)
SMURF2	Induces degradation of SMAD2, TGF-βRI	(65,66)
NEDD4-2	Induces degradation of SMAD2, TGF-βRI	(67)
WWP1	Induces degradation of TGF-βR1	(68)
CHIP	Induces degradation of SMAD1	(69)
JAB1	Induces degradation of SMAD4	(70,72)
ROC1	Induces degradation of SMAD3	(73)
SMAD6	Inhibits phosphorylation of SMAD1/2; competitively binds SMAD4; induces BMPRI degradation; represses BMP-mediated transcription	(74-77)
SMAD7	Inhibits phosphorylation of SMAD2/3; induces degradation of TGF-βRI	(64,66,78)
STRAP	Stabilizes interaction between SMAD7 and TGF-βRI	(72)
BAMBI	Kinase-deficient Type I Receptor decoy	(79)
SKI	Converts nuclear SMAD complex from activating to repressive	(80-82)
SNO	Converts nuclear SMAD complex from activating to repressive	(83,84)
PARP-1	Dissociates SMAD3/SMAD4 complex from DNA	(85)

CHAPTER 2. EFFECTS OF SMAD4 LOSS ON PANCREATIC CANCER

Introduction

SMAD4 was originally identified based on its frequent deletion in pancreatic cancer samples (33) and was later confirmed to be one of the major genes altered in the progression of the disease (21,22). Many studies have been dedicated to gleaning the consequences of SMAD4 loss in pancreatic cancer, both clinically and in model systems. SMAD4 loss has been associated with an increase in metastasis (42) and a corresponding shortening of survival in pancreatic cancer patients (93,94). It makes sense, then, that *in vitro*, SMAD4 loss has been associated with more aggressive behavior (87-90,92).

Curiously, while the downstream members of TGF-β signaling seem to have a clear effect on pancreatic cancer cells, the contribution of upstream components is not as easily discerned. One would assume that loss of TGF-β signaling, in general, would confer a similar result to loss of SMAD4. However, this assumption does not necessarily hold true. Mutation of *TGF-βRII* does not affect prognosis of pancreatic cancer patients, while an elevated presence of TGF-β ligands is associated with reduced survival (93,103). Experimentally, the role of TGF-β in pancreatic cancer has been poorly understood. Supplementing pancreatic cell media with TGF-β promoted epithelial-to-mesenchymal transition and enhanced the production and activity of the pro-metastatic factors matrix-metalloproteinase 2, urokinase plasminogen activator, and urokinase plasminogen activator receptor (95-98). Along these lines, the treatment of pancreatic cancer cell lines and xenografts with a soluble TGF-β receptor II results in reduced invasion *in vitro* and blocks tumor growth, angiogenesis, and metastasis *in vivo* (99,102).

In light of the seemingly contrasting effects of inhibiting SMAD4 and TGF-β receptors in pancreatic cancer, we and others have hypothesized that loss of SMAD4

promotes the switch of TGF-β from a tumor-suppressor to a tumor-promoter (122). It is interesting to speculate that alternative pathways may be activated by TGF-β ligand if the signal is unable to be propagated through its traditional cascade. Several "SMAD-independent" TGF-β pathways have been identified, including Rac, RhoA, CDC42, ERK, JNK, p38, TAK1, NFκB, Pi3K, and Par6 (123-128). It is possible that TGF-β signals are detoured through one of these alternative networks upon loss of SMAD genes in pancreatic cancer. Herein, we describe the effects of SMAD4 loss on pancreatic cancer cells, both in terms of alternative pathway activation and cell behavior.

Materials and Methods

Cell culture. All cell lines were maintained in Dulbecco's Modified Eagle Medium containing 1 g/L glucose (Gibco, Carlsbad, CA), 10% Fetal Bovine Serum (Gemini Bio-Products, West Sacramento, CA), 1% Penicillin/Streptomycin (Quality Biological, Gaithersburg, MD), and 5 μg/mL Plasmocin (Invivogen, San Diego, CA). BxPC3 was purchased from the American Type Culture Collection, while Pa01 and Pa02 were generated in our laboratory.

Cell transfection. All transfections were performed using the Attractene lipofection system (Qiagen, Valencia, CA).

Generation of stably transfected cell lines. Cells were transfected with an empty vector (pcDNA3.1) or a SMAD4 plasmid (pcDNA3.1-DPC4) (obtained from Dr. Scott Kern). Stably transfected cells were maintained by selection with 0.35 mg/mL G418 sulfate. Experiments were performed using either the native transfected cells or cell lines grown from a single cell of the parental isogenic lines.

Polymerase chain reaction. RNA was extracted from cell lines using the RNEasy Mini Kit (Qiagen, Valencia, CA), and five hundred nanograms of RNA was converted to cDNA using the Thermoscript RT-PCR system (Invitrogen, Carlsbad, CA). PCR for *SMAD4* and *β-Actin* was performed using standard methods and the primers listed in Table 2. Amplicons were analyzed by agarose gel electrophoresis.

Luciferase assays. Cells were transiently co-transfected with a construct containing a firefly luciferase reporter under the control of a normal (p6SBE) or mutant Smad-binding element (pm6SBE) in addition to a construct containing a *Renilla* luciferase reporter

(129). 0.01 mg/mL human TGF-β1 (Sigma, St. Louis, MO) was added to the cell medium approximately five hours after transfection. Luciferase activity was measured 45 hours after addition of TGF-β1 using a Wallac 1420 plate reader (Perkin Elmer, Waltham, MA) and normalized to *Renilla* luciferase activity.

Western blotting. Isogenic clones were exposed to 5 ng/mL hTGF-β1 for 24 hours. Cells were then lysed in 1X RIPA buffer containing a cocktail of protease inhibitors. Protein concentration was determined by bicinchoninic acid protein assay (Pierce, Rockford IL), and thirty nanograms of total protein was loaded onto a 4-12% Bis-Tris polyacrylamide gel and transferred onto a nitrocellulose membrane. Antibody conditions are described in Table 3. Signal was detected using the ECL-Plus kit (GE Healthcare, Piscataway, NJ).

Patient samples. Patient samples were obtained from consenting patients undergoing surgical removal of pancreatic cancers at Johns Hopkins Hospital.

Immunohistochemistry. Immunolabeling for p-p44/42 and pAKT was performed using standard methods and the antibody conditions in Table 4. For pAKT, cancer tissue was given an intensity score between 0-3, and cases with a score greater than 2 were deemed "high." For pERK, histology score was calculated. Those cases with a score greater than 100 were considered "high." SMAD4 status was determined by the presence or absence of signal.

Cell proliferation analysis. 2,500 Pa01-Control or Pa01-DPC4 cells/well were seeded in 96-well plates and allowed to adhere overnight. At the time of plating, twenty-four, forty-eight, seventy-two, and ninety-six hours after transfection, cells were treated with 10 μL of CCK8 solution (Dojindo, Rockville, MD) and incubated at 37^{o}C for two hours.

Absorbance at 490 nm was measured using a Wallac 1420 plate reader (Perkin Elmer, Waltham, MA).

Migration and invasion analysis. Migration and invasion were measured by Boyden chamber analysis (BD Biosciences, San Jose, CA). 50,000 Pa01-Control or Pa01-DPC4 cells were plated onto Boyden chambers (untreated for migration, covered in matrigel for invasion) in media containing 0.5% FBS. After 24 hours, the inserts were subjected to a 0.5% to 10% FBS gradient. Cells were allowed to migrate or invade through matrigel for 48 hours. At the endpoint of the experiment, cells having migrated or invaded were fixed and stained using Diff-Quik (Siemens, Deerfield, IL). Cells were counted in three independent fields per chamber using an inverted microscope at 10X magnification.

Statistical analysis. Statistical analyses were performed using SISA, Microsoft Excel, and the R environment.

Results

Establishment of Cell Lines Stably Complemented with SMAD4

In order to study the effect of SMAD4 loss on pancreatic cancer biology, we sought to generate stable isogenic cell lines (control and SMAD4-complemented) using three parental cell lines, Pa01, Pa02, and BxPC3, all of which contain homozygous deletions of *SMAD4* (21,130). Cells were transfected either with an empty vector ("Control") or a plasmid containing full-length, wild-type SMAD4 ("DPC4") and selected with G418 sulfate (131). The generation of stable cell lines was confirmed using semi-quantitative PCR for *SMAD4* transcript (Figure 2A), as well as a luciferase assay for TGF-β activity (Figure 2B). *SMAD4* transcript was detected in two out of the three cell lines attempted: we were successfully able to complement SMAD4 into Pa01 and BxPC3, but not Pa02 (Figure 2A). Despite successfully complementing SMAD4 into Pa01 and BxPC3, the change in TGF-β response in these cell lines is minimal (Figure 2B). Thus, we can infer that, either, an additional factor is blocking TGF-β activity in these cell lines or that there is heterogeneity among these cells in regards to the transfection efficiency. These observations will be addressed further in Chapter 4.

SMAD-independent pathways and their relationship with SMAD4 in pancreatic cancer.

Much of what we understand of TGF-β signaling is through analyses of the canonical, SMAD-dependent cascade. However, TGF-β ligands have been shown to be more promiscuous in their downstream effectors. TGF-β can activate small GTPase

pathways (Rac, RhoA, and CDC42) (123,124), mitogen-activated protein kinase pathways (ERK, JNK, and p38) (123-125), NFκB (126), Pi3K (127), and Par6 (128).

Given the prior observations that modulation of TGF-β receptor levels cause the opposite phenotype than modulation of SMAD4 (93,95-99,102,103), we hypothesized that these receptors act independently, to some degree, of their canonical downstream mediators. For example, in pancreatic cancers where SMAD4 is lost, it is conceivable that TGF-β ligand will stimulate its receptors to signal down an alternative pathway. To address this possibility, we analyzed the activity of several SMAD-independent pathways in our SMAD4-isogenic cell lines. As shown in Figure 3, the p44/42 mitogen-activated protein kinase pathway (ERK) and Pi3K/AKT pathways are both preferentially activated in the absence of SMAD4. It is worth noting that the activation of ERK in Pa01 was TGF-β ligand-independent, whereas it was somewhat ligand-dependent in BxPC3 cells. Genetically, the major difference between these two cell lines is the maintenance of wild-type *KRAS* in BxPC3, which is an upstream activator of ERK signaling (23). This genetic disparity may explain the difference in pathway activation between the two cell lines.

Given this interesting observation regarding the preferential activation of ERK and AKT in the absence of SMAD4, we sought to verify these data in pancreatic cancer patients. To this end, we performed immunohistochemistry for pERK and pAKT in surgically resected primary pancreatic cancers with known SMAD4 status. As shown in Table 5 and Table 6, there is no significant association between ERK or AKT activity and SMAD4 status in this cohort.

The effects of SMAD4 restoration on pancreatic cancer cell behavior.

Given the association between SMAD4 loss and increased metastasis in pancreatic cancer (42), we sought to determine a rationale, *in vitro*, for these observations. We generated single cell-derived clones from the pooled isogenic cell lines established above. In particular, we focused our studies on the Pa01 cell line, which was established from a liver metastasis at rapid autopsy. The clones chosen for further analysis retain expression of the *SMAD4* transcript (Figure 4A) and have restored TGF-β activity to a greater degree than their parental cell lines (Figure 2B, Figure 4B), suggesting that there is heterogeneity among the parental lines.

The prior observations that SMAD4 activates cell cycle inhibitors, such as *p21/waf1* and *p15/ink4b*, indicates that suppression of cell proliferation is, potentially, a major function of the gene (87,88). Indeed, as shown in Figure 5, the degree to which SMAD4-complemented cells proliferate is significantly reduced compared to SMAD4-negative cells, as expected. Given that SMAD4 loss was associated with widespread metastasis in pancreatic cancer patients (42), we sought to determine the effects of SMAD4 modulation on the degree to which pancreatic cancer cells migrate and invade *in vitro*. Previous work has suggested that SMAD4 does suppress the TGF-β-induced migration and invasion of pancreatic cancer cells (132). We were able to verify the link between SMAD4 and cell mobility, although from a cell autonomous perspective: restoration of SMAD4 in Pa01 cells dramatically reduced the ability of the cells to migrate (Figure 6; $p<0.0001$) and invade through matrigel (Figure 6; $p<0.0001$).

Discussion

Since its discovery, SMAD4/DPC4 has been extensively studied in the context of pancreatic cancer. Both in cell culture models and clinically, SMAD4 loss has been associated with more aggressive pancreatic cancer (42,87,88,92-94). Herein, we have confirmed, using an isogenic cell model, the effect of SMAD4 on pancreatic cancer cell proliferation (Figure 5), migration (Figure 6A), and invasion (Figure 6B). Furthermore, our results suggest that alternative, SMAD-independent pathways, such as ERK and Pi3K, may be preferentially activated in the absence of SMAD4 (Figure 3).

Our results show a clear effect of SMAD4 loss on pancreatic cancer cell behanior. We have validated prior work showing that SMAD4 loss promotes proliferation of pancreatic cancer cells (86-88) (Figure 5), while we have shown a cell-autonomous effect of SMAD4 loss on migration (Figure 6A; $p<0.0001$) and invasion (Figure 6B; $p<0.0001$). These *in vitro* data support many clinical studies noting an association between SMAD4 status and pancreatic cancer prognosis and metastatic burden (42,93,94). Further study will attempt to clarify the mechanism through which SMAD4 loss contributes to the increased aggressiveness of pancreatic cancer, both *in vitro* and *in vivo*.

One possibility that we considered was the activation of SMAD-independent TGF-β pathways upon SMAD4 loss. Exposing pancreatic cancer cells to TGF-β ligand results in epithelial-to-mesenchymal transition and the production of pro-metastatic signals, while blocking all TGF-β signaling is protective against tumor growth *in vitro* and *in vivo* (95,96,96-99,102). Given that TGF-β has previously been shown to activate pathways other than its canonical SMAD network, we hypothesized that, perhaps, in lieu

of completely blocking a TGF-β response, SMAD4 loss causes the redirection of the TGF-β signal through another pathway. Through immunoblotting of cell lines isogenic for SMAD4 in the presence or absence of TGF-β1, we were able to associate the activation of the ERK and Pi3K pathways with SMAD4 loss (Figure 3). Both of these pathways have previously been implicated in the development of pancreatic cancer. Inhibition of ERK signaling has been associated with a cell cycle arrest and, possibly, increased apoptosis in pancreatic cancer cell lines (133,134). Active AKT is also a survival factor in pancreatic cancer, while is also promotes invasiveness and is associated with more poorly differentiated disease (135-137). However, despite our observations in cell culture, we were not able to associate ERK or Pi3K activation with SMAD4 status in primary pancreatic cancers (Table 5, Table 6). Further study will clarify if there is an alternative TGF-β network that is selected for in SMAD4-negative pancreatic cancers, other than ERK and Pi3K, that would contribute to the progression of pancreatic cancer upon SMAD4 loss.

Table 2. Primer sequences used for semi-quantitative PCR.

Primer Name	Primer Sequence
SMAD4 Forward	5-TAGGCAAAGGTGTGCAGTG-3
SMAD4 Reverse	5-CGATGACACTGACGCAAATC-3
β-Actin Forward	5-GACCCAGATCATGTTTGAGAC-3
β-Actin Reverse	5-GATGGGCACAGTGTGGGTGAC-3

Table 3. Antiobdies used for immunoblotting.

Antibody	Company	Dilution
p-p44/42	Cell Signaling (Danvers, MA)	1:1000
t-44/42	Cell Signaling (Danvers, MA)	1:2000
pAKT	Cell Signaling (Danvers, MA)	1:500
AKT	Cell Signaling (Danvers, MA)	1:1000
GAPDH	Cell Signaling (Danvers, MA)	1:5000
HRP-linked anti-rabbit IgG	GE Healthcare (Piscataway, NJ)	1:5000

Table 4. Antibodies used for immunohistochemistry.

Antibody	Company	Dilution
p-p44/42	Cell Signaling (Danvers, MA)	1:100
pAKT	Cell Signaling (Danvers, MA)	1:100
SMAD4	Santa Cruz Biotechnology (Santa Cruz, CA)	1:100

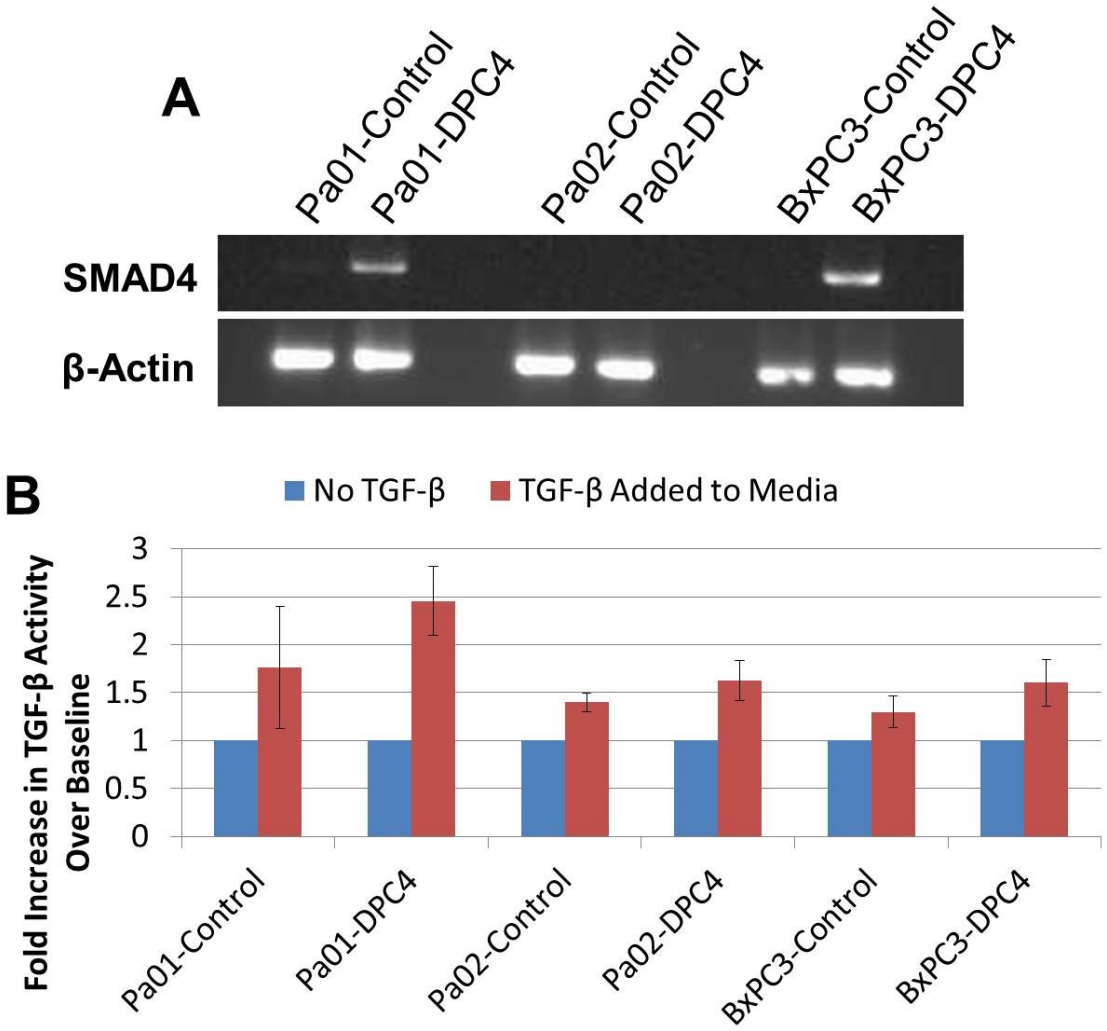

Figure 2. Complementation of SMAD4 into Pa01, Pa02, and BxPC3.
Pa01, Pa02, and BxPC3 cells were stably transfected with either an empty vector (pcDNA3.1) or a DPC4 expression vector (pcDNA3.1-DPC4). A) Only Pa01-DPC4 and BxPC3-DPC4 express *SMAD4* (Pa02-DPC4 cells do not), whereas the corresponding control cells do not. B) Isogenic cell lines were analyzed using a luciferase screen for TGF-β response. Data is presented such that the wild-type to mutant promoter ratio is set to 1 for all cell lines (baseline). TGF-β activity is presented as the fold increase in luciferase levels after TGF-β addition to media (SBE + TGF-β/mSBE + TGF-β). Error bars represent standard error of the mean.

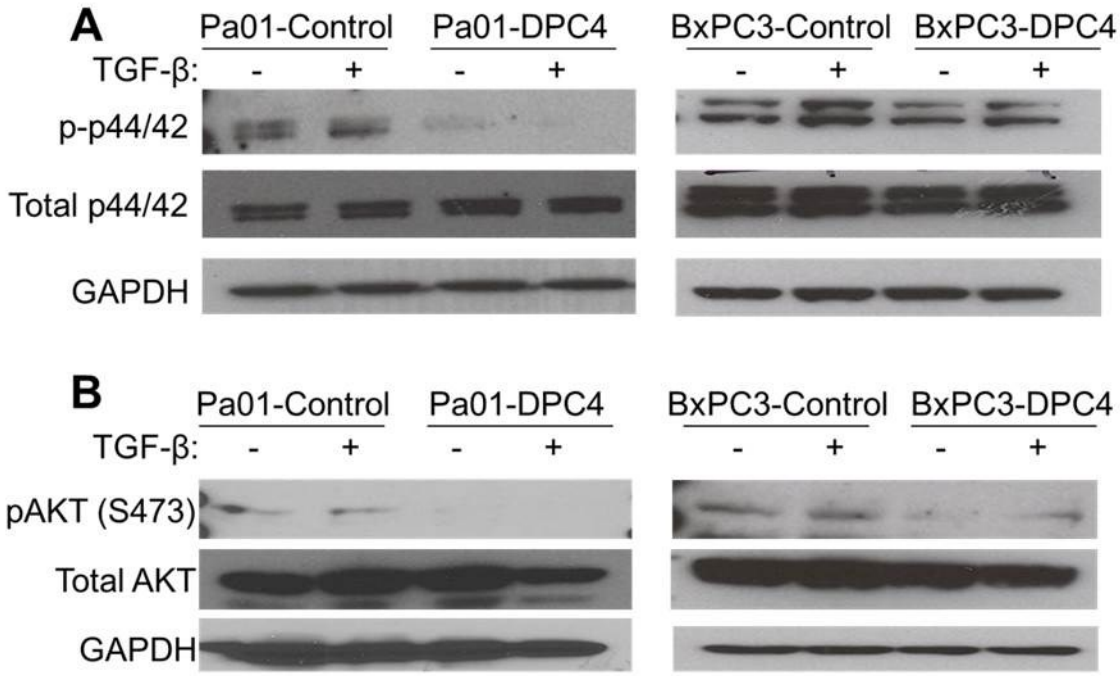

Figure 3. Effect of SMAD4 restoration on SMAD-independent TGF-β pathways. Cells were treated with 5 ng/mL h-TGF-β1 for 24 hours or left untreated. A) Analysis of p-p44/42 (ERK) signaling activity. B) Analysis of pAkt signaling activity.

Table 5. Relationship between SMAD4 status and ERK pathway activity in primary pancreatic cancers.

	Low p-p44/42 (N=23)	**High p-p44/42 (N=24)**	**p**
SMAD4 Status			
Intact	16	12	0.17
Lost	7	12	

* Significance was calculated using Chi-square analysis.

Table 6. Relationship between SMAD4 status and AKT pathway activity in primary pancreatic cancers.

	Low pAkt (N=36)	**High pAkt (N=11)**	**p**
SMAD4 Status			
Intact	22	6	0.48
Lost	14	5	

* Significance was calculated using Fisher exact analysis.

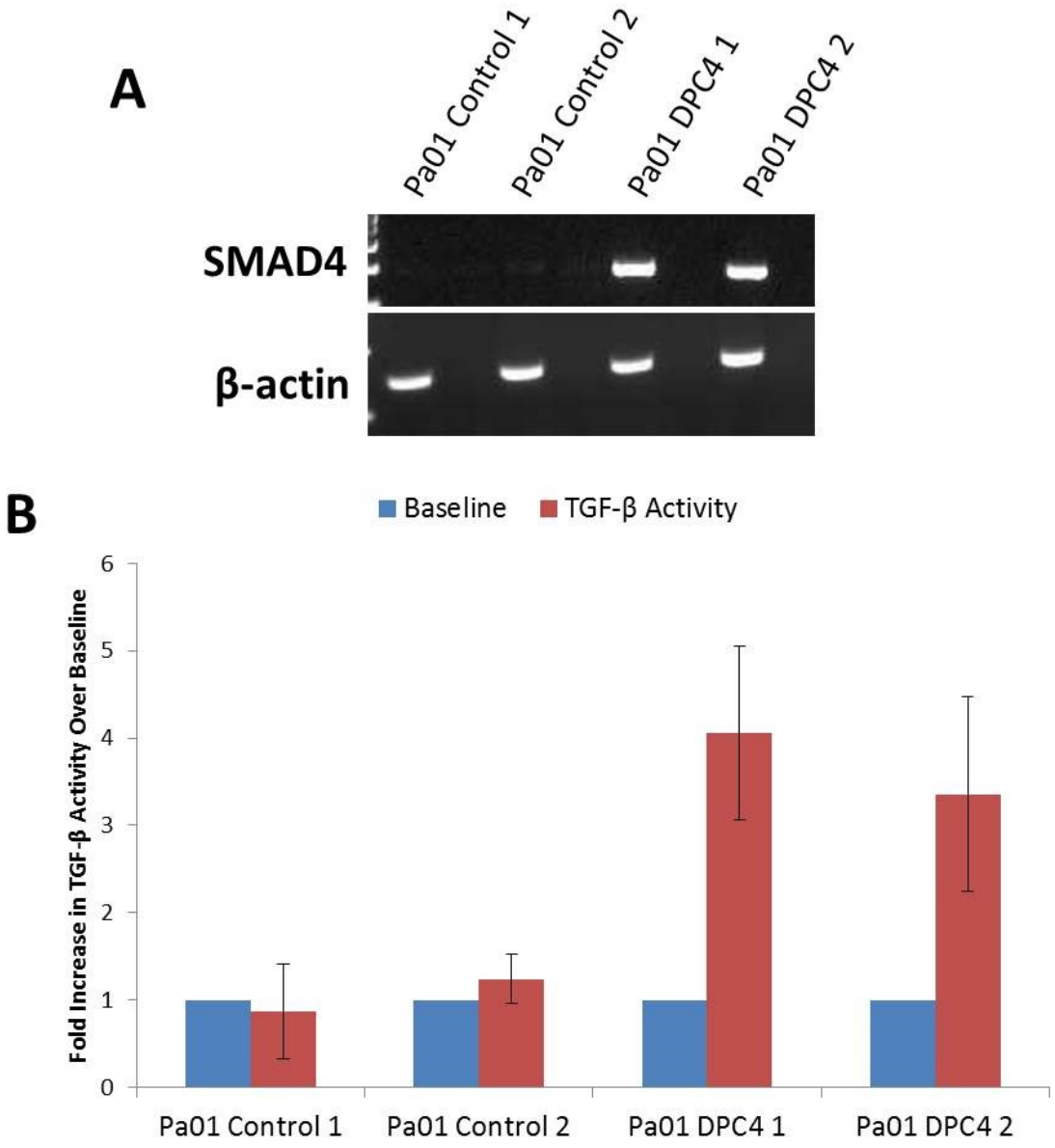

Figure 4. TGF-β responsiveness in single-cell SMAD4-isogenic clones.
Isogenic cell lines were grown from single cells of the parental Pa01 Control and Pa01 DPC4 cell lines (Figure 2) and analyzed using semi-quantitative PCR for *SMAD4* (A) and a luciferase screen for TGF-β response (B). Data is presented such that the wild-type to mutant promoter ratio is set to 1 for all cell lines (baseline). TGF-β activity is presented as the fold increase in luciferase levels after TGF-β addition to media (SBE + TGF-β/mSBE + TGF-β). Error bars represent standard error of the mean.

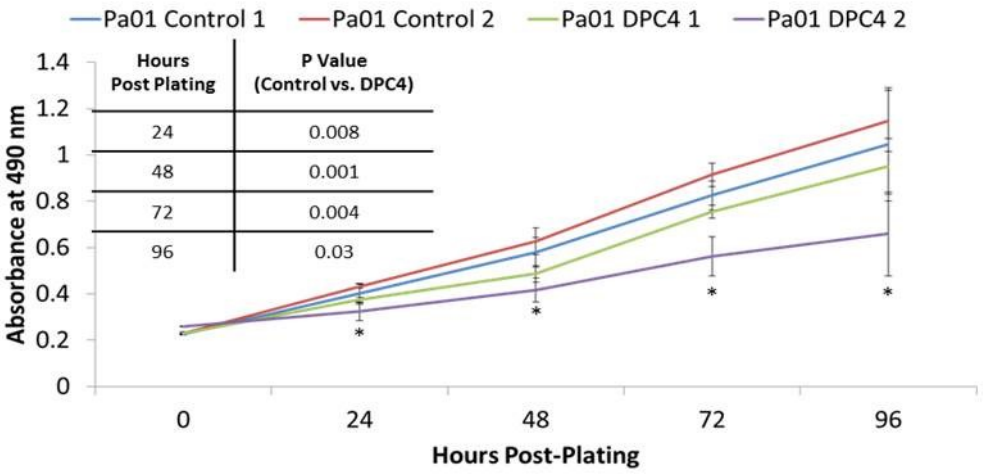

Figure 5. The impact of SMAD4 complementation on cell viability.
Restoration of SMAD4 reduces proliferation in Pa01 cells as soon as one day after plating. Statistical significance was determined by a contrasted ANOVA. Error bars represent standard deviation.

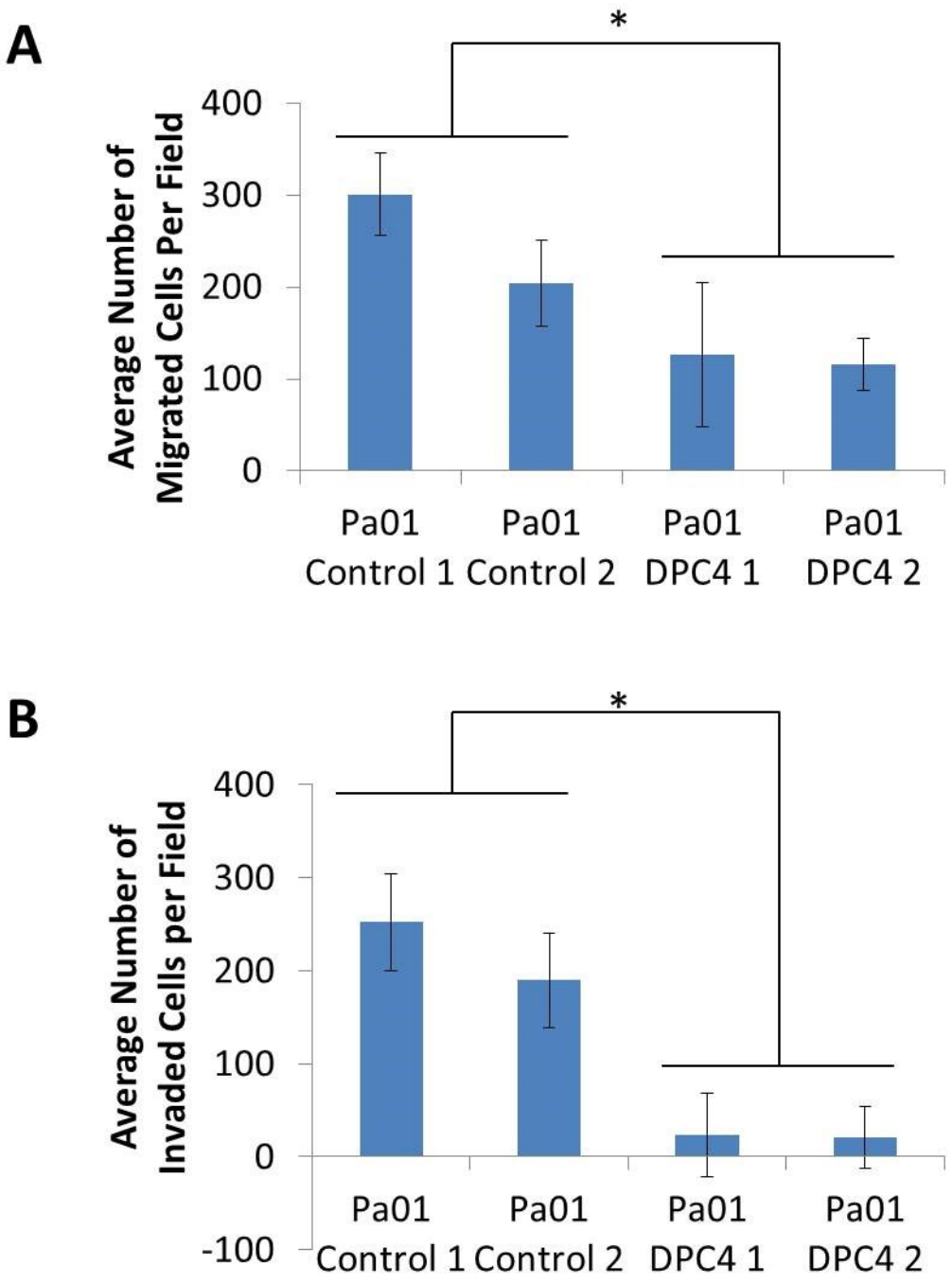

Figure 6. The effect of SMAD4 complementation on cell migration and invasion. A) Restoration of SMAD4 reduces migration in Pa01 cells ($p<0.0001$). Error bars represent standard deviation. B) Complementation of SMAD4 reduces invasion through matrigel in Pa01 cells ($p<0.0001$). Error bars represent standard deviation. Significance of the differences between Control and DPC4 cells was calculated using a Kruskal-Wallis test.

CHAPTER 3. SMAD4 CONFERS DIFFERENTIAL CHEMOSENSITIVITY ON PANCREATIC CANCER CELLS *IN VITRO*[1] [2]

[1] This chapter stems from a collaborative paper between our laboratory and the laboratory of Dr. James Eshleman, led by Yunfeng Cui.

[2] Adapted by permission from the American Association for Cancer Research: Cui et al., Genetically defined subsets of human pancreatic cancer show unique *in vitro* chemosensitivity, Clinical Cancer Research, 2012, 18:23, 5619-6530. (131)

Introduction

Given the dire prognosis associated with pancreatic cancer, there has been significant interest in developing more effective means to treat the disease. To date, gemcitabine remains the standard of care for pancreatic cancer, but is not particularly effective on its own; although, its efficacy increases when combined with erlotonib and Nab-paclitaxel (9-11). In addition, the FOLFIRINOX and GTX regimens have shown preliminary effectiveness (12,13).

The strategy of treating cancer based on its genetic profile has shown promise in other cancer types, notably breast cancer and chronic myeloid leukemia (138-140). In pancreatic cancer, success in personalized medicine has, largely, evaded us. A recent study failed to identify synthetically lethal interactions with oncogenic *KRAS* in pancreatic cancer cells (141). However, a familial pancreatic cancer harboring a *PALB2* mutation was shown to be susceptible to DNA-damaging agents cisplatin and mitomycin C, suggesting that the genetic-based guidance of pancreatic cancer therapy is not an insurmountable hurdle (142). Furthermore, preliminary data suggest that PARP inhibitors may prove effective in treating familial pancreatic cancers with *BRCA2* mutations, although the translation of this concept to the clinic remains (143-145).

We were interested in determining the effect of the major driver mutations (*KRAS*, *CDKN2A*, *TP53*, and *SMAD4*) on the effectiveness of chemotherapeutics in pancreatic cancer cell lines. We took advantage of the availability of cell lines with known genotypes (21) and cell lines isogenic for SMAD4 (described in Chapter 2). Herein, we, in conjunction with the laboratory of Dr. James Eshleman, describe our findings relating SMAD4 status to the effectiveness of several chemotherapeutic agents.

Materials and Methods

Human PC cell lines, human pancreatic ductal epithelial cell line, human DPC4/SMAD4 isogenic PC cell lines, and cell culture. Thirty-four human pancreatic cancer cell lines used in this study were isolated from the tumor samples of patients in Departments of Pathology and Oncology, Johns Hopkins School of Medicine. Among them, nineteen cell lines were used for the Discovery Screen and ten cell lines were used for the Prevalence Screen of the original PC sequencing study (21). The sequences of 23,219 transcripts representing 20,661 protein-coding genes were determined, and somatic mutations identified in the discovery and prevalence screen, homozygous deletions, and SAGE gene expression data have been published in detail elsewhere (21). Twelve of the patients contributing samples to this study had a family history of the disease, defined as having at least two first degree relatives affected with pancreatic cancer. The use of human tissue was approved by the local IRB (John Hopkins University). All human pancreatic cancer cell lines were cultured in MEM medium (Gibco, Grand Island, NY) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco), 100 U/ml Penicillin and 100 ug /ml Streptomycin.

An HPV transfected normal human pancreatic ductal epithelial cell line (HPDE), generously provided by Dr. Ming-Sound Tsao, was also analyzed. Apart from slightly aberrant expression of p53, molecular profiling of this cell line has shown that expression of other proto-oncogenes and tumor suppressor genes are normal (146). The HPDE cell line was grown in keratinocyte serum free-media (Invitrogen, Grand Island, NY) supplemented with bovine pituitary extract and recombinant epidermal growth factor.

Pa01C and Pa02C parental cell lines were both generated from liver metastases of PC patients, which both had homozygous deletions of DPC4. Pa01C and Pa02C cells were stably transfected with either a cDNA expression plasmid to overexpress DPC4 (pcDNA3.1-DPC4) or an empty plasmid (pcDNA3.1Mock) using the Attractene transfection agent (Qiagen, Valencia, CA). The Pa01C and Pa02C derivative isogenic cells were both analyzed as single clones. All of the genetically complemented clones express DPC4, while the control cells do not (Figure 4A). TGF-β pathway activities were tested for these isogenic pairs using a previously reported luciferase assay (129). These cell lines were cultured in DMEM medium (Gibco) supplemented with 10% FBS (Gibco), 100 U/ml Penicillin, 100 ug /ml Streptomycin, and 0.35 mg/mL G418 sulfate (Mediatech, Manassas, VA). All cells were maintained at 37 ℃ in a humidified atmosphere with 5% CO_2.

Preparation of anticancer drugs. Triptolide, docetaxel, MMC, cisplatin, irinotecan and artemisinin were purchased from Sigma (St Louis, MO). Gemcitabine was obtained from Net Qem (Research Triangle Park, NC). Parp1 inhibitor (KU0058948) was synthesized in Dr. Vogelstein's lab. Artemisinin (an anti-malarial drug) was selected as a negative control drug since it possessed no known anticancer activity. The eight drugs were dissolved in DMSO (triptolide, docetaxel, cisplatin, irinotecan and artemisinin) or PBS (MMC and gemcitabine), stock solutions of 10mM were made and they were stored at -80 ℃.

In vitro cell growth assay for drug screening. Cells were plated in 96-well plates at a density of 3,000 cells per well using standard culture media (described above). Edge wells of the 96-well plate were filled with 1 X PBS. After overnight growth, media were

removed and 200 µl of medium with drug was added into each well. Drugs were serially diluted to desired final concentrations with medium containing 10% serum and each dose had 6 replicate wells. Cells incubated in the medium with the vehicle DMSO or PBS served as controls. Plates were incubated with drugs for 72 hours. Media were aspirated and each well was washed with PBS 3 times to remove cellular debris. 100 µl of ddH_20 was added to each well and plates were incubated at room temperature for 1 hour to lyse the cells. 100 µl of deionized H_2O containing 0.15% of SYBR green I solution (Molecular Probes, cat # S7567) were added to wells and mixed 10 times. Fluorescence was measured by using BMG FluoStar Galaxy (BMG Labtechnologies, USA)(excitation at 480 nm and measurement at 520nm) (147).

Human PC cell lines and HPDE were tested initially at the following doses: 0 nM, 1 nM, 10 nM, 100 nM, 1 µM, 10 µM and 100 µM. Positive drug effects and isogenic cells drugs were tested using final concentrations: 0 nM, 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 µM, 3 µM and 10 µM in three independent experiments (18 replicate wells totally per dose).

Statistical analysis. IC50 values were calculated by fitting sigmoid dose-response curves with GraphPad Prism 5.0 (GraphPad Software, Inc.). IC50 distributions were compared by genetic status (mutations, deletions or both) and family history status with Wilcoxon rank sum tests. A drug response was defined as an IC50 value below the median or below the highest IC50 that did not exceed 10,000 (this happened for drugs where more than 50% of cell lines had IC50 values greater than or equal to 10,000). The relationship between response and genetic status was summarized with an odds ratio from a logistic

regression model and Fisher's exact test. When comparing the IC50 values between different drugs or to fold change in pathway activity, Spearman's rank based correlation was used. IC50 values between isogenic pairs were compared with a paired Student's t test. Chemosensitivity correlation analysis of clustered cell lines was examined with heatmaps and a network map, which is a visual depiction of how cell lines cluster based on whether the Spearman correlation of the within-cell line standardized IC50 values between a pair of cell lines was 0.65 or higher. Statistical analyses were performed using GraphPad Prism 5.0 and R (version 2.13.1). Due to the exploratory nature of the study, p-values are not corrected for multiple comparisons and are included for descriptive purposes only.

Results

Substantial variation of PC cell lines to broad classes of chemotherapeutic agents

We screened 29 genetically defined pancreatic cancer cell lines with the seven broad classes of anticancer drugs listed above. Pancreatic cancer cells were exposed to increasing concentrations of the drugs in 6 replicate wells and IC50 values calculated (Figure 7). Most pancreatic cancer cell lines were sensitive to gemcitabine, triptolide, docetaxel and MMC. The majority of pancreatic cancer cell lines were insensitive to cisplatin, irinotecan, Parp1 inhibitor and the negative control drug. Among the generally sensitive drugs, some cell lines were notably less sensitive (Figure 8). For example, with gemcitabine, the vast majority of cell lines were sensitive with IC50s in the single digit nanomolar range, whereas three cell lines (Pa09C, Pa08C and Pa43C) were significantly less sensitive (IC50s: 40 to 60 nM). Surprisingly, among the generally ineffective drugs, individual cell lines were uniquely sensitive (Figure 9). For example, with cisplatin, most cell lines were quite insensitive, whereas a few (Pa18C, Pa21C and Pa228C) had relatively lower IC50 values (100 to 200 nM). Similarly, for irinotecan, three cell lines (Pa21C, Pa37C and Pa227C) demonstrated IC50 values less than 200nM.

DPC4/SMAD4 inactivation sensitized PC cells to cisplatin and irinotecan but reduced their sensitivity to gemcitabine

We attempted to explain the variation in drug response by correlating it with the genetic status of these lines. We checked all the somatic mutations and homozygous deletions which existed in the 19 pancreatic cancer cell lines included in the discovery

screen and the 10 pancreatic cancer cell lines included in the prevalence screen (21). We attempted to correlate drug responses to genotype (Figure 10 and Figure 11), considering each gene as either mutated only (mut), deleted only (del), mutated or deleted combined (md), or wildtype (wt). We initially focused on DPC4/SMAD4.

Pancreatic cancer cells with DPC4 homozygous deletions were modestly (2-fold) more sensitive to cisplatin compared to those with wild type genotype (P=0.04; Figure 11 and Table 7). Cells with the deletion of any of four SMAD Pathway genes (*SMAD3*, *SMAD4*, *TGF-βR2* or *TGF-βR3*) were 2.2-fold more sensitive to cisplatin (P=0.02; Table 7). In logistic regression analysis, comparisons of DPC4 deleted or SMADPath gene defective (mutated or deleted) PC cells to wildtype cells treated with cisplatin confirmed this finding (odds ratios: 11.93 and 7.56, P values: 0.01 and 0.05, respectively; Table 8).

One concern about this study is that of multiple comparisons. Accordingly, to confirm these findings, we constructed isogenic cell lines from Pa01C with or without homozygous DPC4 deletions (Figure 4). With cisplatin, we found that IC50 values of DPC4 deficient cell lines for cisplatin were 1.8-fold and 1.2-fold lower than for DPC4-containing cell lines (Figure 12A and Table 9). This was less significant in another isogenic cell line pair derived from Pa02C, but this cell line showed lower TGF-β signaling even when DPC4 was present (Figure 13). Because all the pairs of isogenic cells had different levels of TGF-β pathway signaling (Figure 13A), we attempted to correlate the cisplatin IC50 values with TGFβ pathway activities in these cells. Our results confirmed that IC50 values of cisplatin were positively correlated with TGF-β activity (r=0.86, 95%CI: 0.39 to 0.97, P=0.007), although this relationship is largely driven by the two cell lines with highest TGF-βR activity (Figure 13 and Table 10).

Pancreatic cancer cells with DPC4 defects (mutations or deletion combined) were 4.5-fold more sensitive to irinotecan than those with wildtype DPC4 (P=0.04; Figure 10 and Table 7). In logistic regression analysis, comparison of DPC4 defective cells to wildtype cells treated with irinotecan produced an odds ratio of 8.64 and a P value of 0.02, and when only DPC4 deleted cells were compared to wildtype cells, a P value of 0.02 was obtained (Table 8). In order to verify these findings, we tested the pairs of DPC4 isogenic cell lines with irinotecan. We found IC50 values of DPC4 deficient cell lines were 1.2-fold and 1.7-fold lower than those of DPC4 complemented cell lines (Figure 12B and Table 9). Correlation analysis for IC50 values and TGF-β pathway activities was not significant (P=0.067; Table 10).

We tested two pairs of DPC4 isogenic cells for possible synergy between cisplatin and irinotecan at a constant ratio of 1:1 with simultaneous treatment. The combination indices (CI) were calculated using CompuSyn (26). Simultaneous treatment with the two drugs resulted in antagonistic interactions with DPC4 defective cells and synergistic interactions in DPC4 complemented clones at ID50, but these cell lines all showed antagonistic or additive interactions at ID75 (Table 11).

Despite the lack of correlation between DPC4 status and gemcitabine response in the panel of pancreatic cancer cell lines (Figure 11), we tested the DPC4 isogenic cell lines with gemcitabine, and found that DPC4 defective cells were about 2-fold less sensitive than wildtype cells (P=0.058 and 0.033; Figure 12C and Table 9). We made the correlation analysis for IC50 values and TGF-β pathway activities in these isogenic cells. Our results confirmed that gemcitabine IC50s were negatively correlated with TGF-β pathway activities (P=0.015; Figure 13C and Table 10).

Discussion

As the cost and ease of sequencing falls, the interest in developing personalized medicine schema has been rising. As such, herein, we sought to determine the relationship between SMAD4 genetic status and chemosensitivity *in vitro*, in the hopes of establishing SMAD4 as a potential clinical marker to guide treatment. These studies were performed in collaboration with Dr. Yunfeng Cui and Dr. James Eshleman.

Upon a screen of the cell lines that underwent whole-exome sequencing by Jones *et al.*, variability was observed in the sensitivity of cell lines to chemotherapeutics *in vitro* (21,131) (Figure 7, Figure 8, Figure 9). Because of the frequency at which alterations in SMAD4 occur in pancreatic cancer, we were intrigued by the possibility that SMAD4 status could correspond to therapeutic efficacy of these chemotherapeutic agents. Indeed, the differential sensitivity to cisplatin and irinotecan among the cell lines described above was due, in some part, to differences in SMAD4 status (Figure 10, Figure 11, Table 7, Table 8).

The single-cell derived isogenic cell lines described herein were utilized to better understand the relationship between SMAD4 and therapeutic efficacy. SMAD4-complemented cells were less sensitive to cisplatin ($p=0.04$, Figure 12A, Table 9) and irinotecan ($p=0.03$, Figure 12B, Table 9) than SMAD4-null cells, while SMAD4-complemented cells were more sensitive to gemcitabine ($p=0.03$, Figure 12C, Table 9). While these associations were statistically significant, the changes in IC50 values between SMAD4-intact and –null cell lines were small. Still, these data are an important

proof of principle that a patient's genotype can potentially guide treatment of his or her disease.

Furthermore, for cisplatin and gemcitabine, in particular, the degree to which the isogenic cells were sensitive or resistant to the drugs was correlated to the degree of TGF-β pathway activity (Figure 13, Table 10). These data indicate that factors other than SMAD4 may influence the sensitivity of pancreatic cancer cells to these agents, as well. It is worth noting that TGF-β signaling was identified as being one of the most commonly altered pathway in pancreatic cancer via whole-exome sequencing, although the causative mutations differed from patient to patient (i.e. mutations in other SMAD pathway members were identified) (21). As such, it is not surprising that mutations in *TGF-βRII* also conferred resistance to irinotecan ($p=0.03$, Table 7), while mutations in any TGF-β pathway member conferred resistance to cisplatin ($p=0.02$, Table 7). Given that SMAD4 status, clinically, is typically determined by immunohistochemistry rather than sequencing (35), SMAD4 is a good candidate for pre-therapy patient stratification. However, given the relationship between TGF-β activity and cisplatin and gemcitabine efficacy, one must wonder about the degree to which the presence of SMAD4 is indicative of pathway activation. Previous studies have identified a lack of TGF-β response in cell lines identified as being wild-type for pathway members as well as in cell lines complemented with SMAD4, as herein (148,149). This issue will be addressed further in the next chapter.

In addition to our studies that correlate SMAD4 status to differential chemosensitivity *in vitro*, recently, independent studies with SMAD4-isogenic cell lines have identified two novel compounds, UA62001 and UA62784, which preferentially

target cells lacking SMAD4 (150,151). These results, certainly, validate our hypothesis that SMAD4 status can theoretically be used to stratify patients into certain treatment groups. The benefit to our study is that the agents analyzed are already used in the clinic. However, there are limitations to all three studies in that they are solely conducted *in vitro*. Further experimentation in animal models of pancreatic cancer and eventual clinical trials will be necessary to fully understand if stratification by SMAD4 status is an appropriate clinical strategy for treatment of pancreatic cancer. Nonetheless, it is encouraging that the age of personalized medicine in cancer therapy appears to be rapidly approaching.

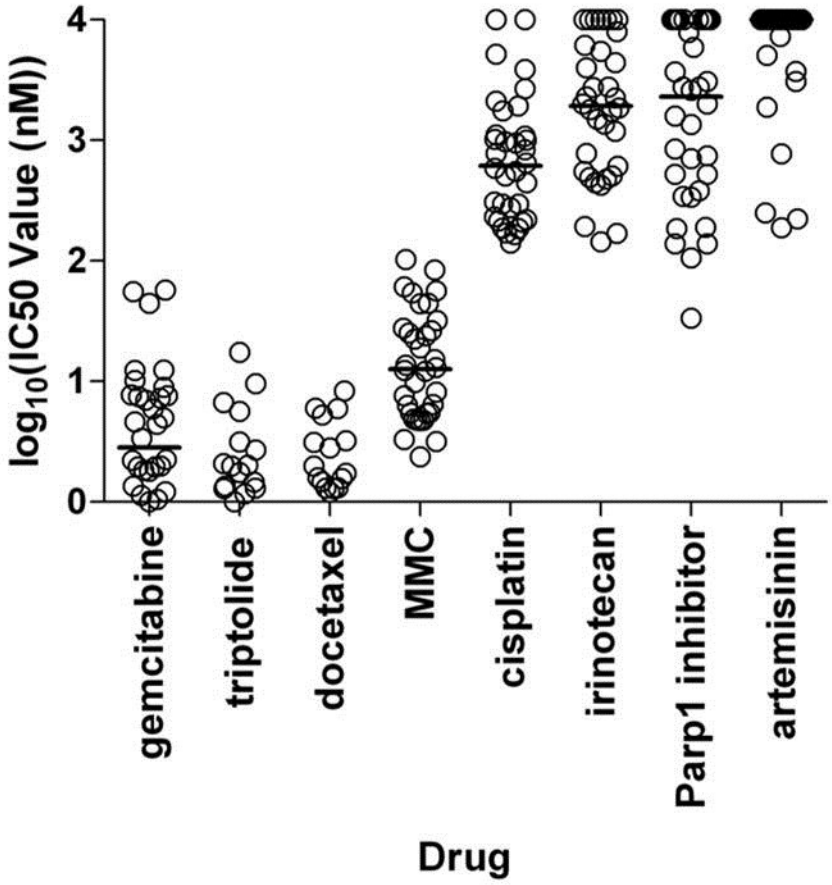

Figure 7. Chemosensitivity of PC cell lines to broad classes of chemotherapeutic agents.

Drug responses (Log_{10} of the IC50 values) of 34 PC cell lines tested with 7 chemotherapeutic drugs representing different mechanisms of action, and the control drug (artemisinin). Each cell line result is represented by a single circle. Horizontal lines indicate the median IC50 value for each drug for all cell lines. The four drugs on the left are generally sensitive drugs, and the four drugs on the right are generally inactive.

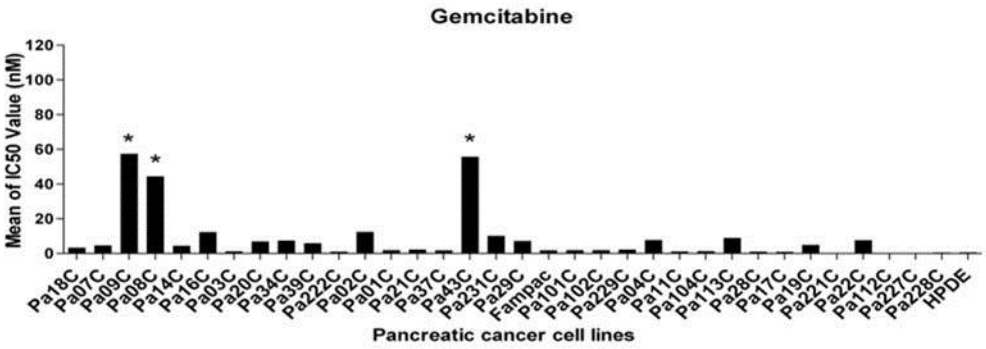

Figure 8. Responses of individual PC cell lines to gemcitabine.
Cytotoxic effects of gemcitabine on PC cells. Gemcitabine demonstrated generally good responses, although unique cell lines showed more than 100 fold less sensitivities (* indicates outlier cell lines).

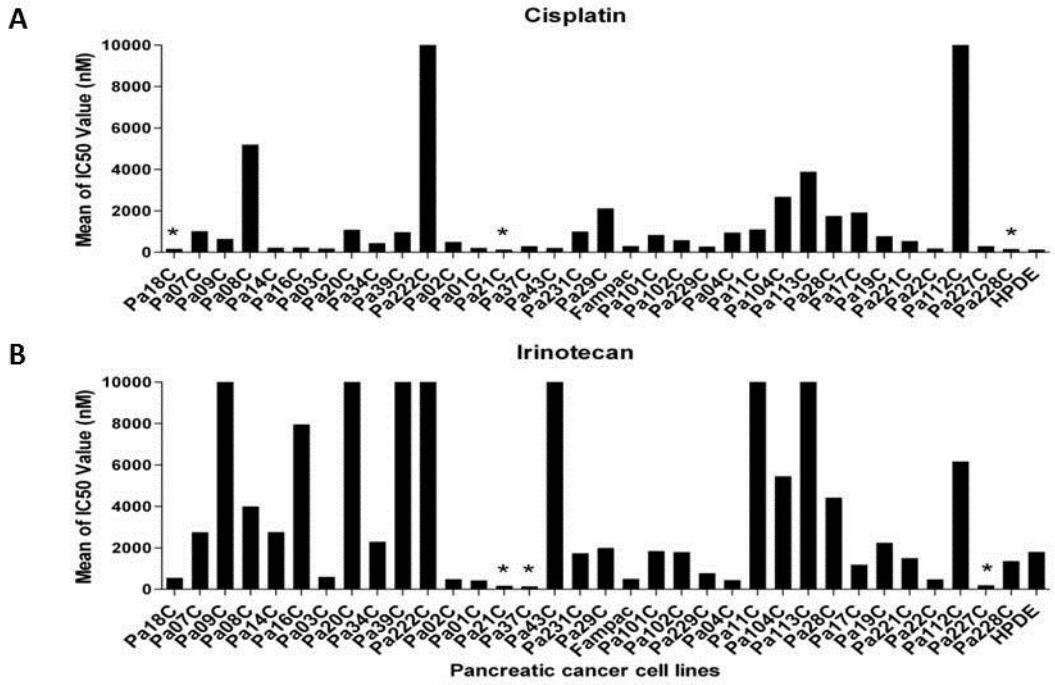

Figure 9. Responses of individual PC cell lines to insensitive drugs.
Cytotoxic effects of cisplatin (A), irinotecan (B) on pancreatic cancer cell viability demonstrated variable drug responses, and unique cell lines showed more than 200 fold variation in their sensitivities to these drugs (*indicates uniquely sensitive cell line).

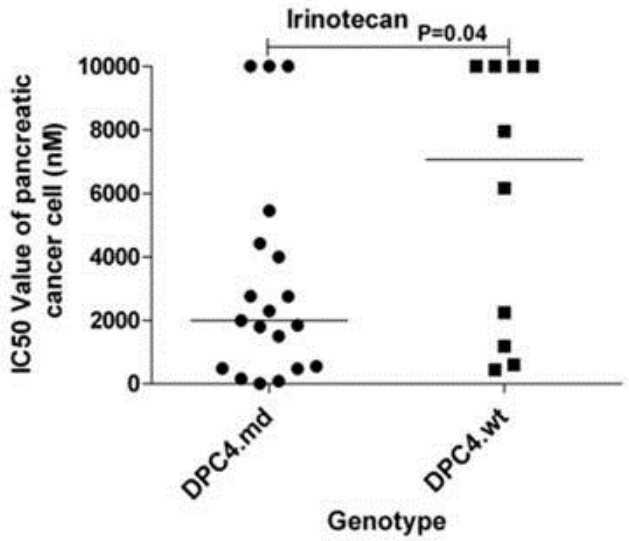

Figure 10. Correlations of drug response to genotype.
Cytotoxic effect of irinotecan was related to DPC4/SMAD4 inactivation. Wilcoxon rank sum tests were used to compare differences in the median IC50 by mutation status. "md" indicates either mutation or deletion and the "wt" indicates wildtype.

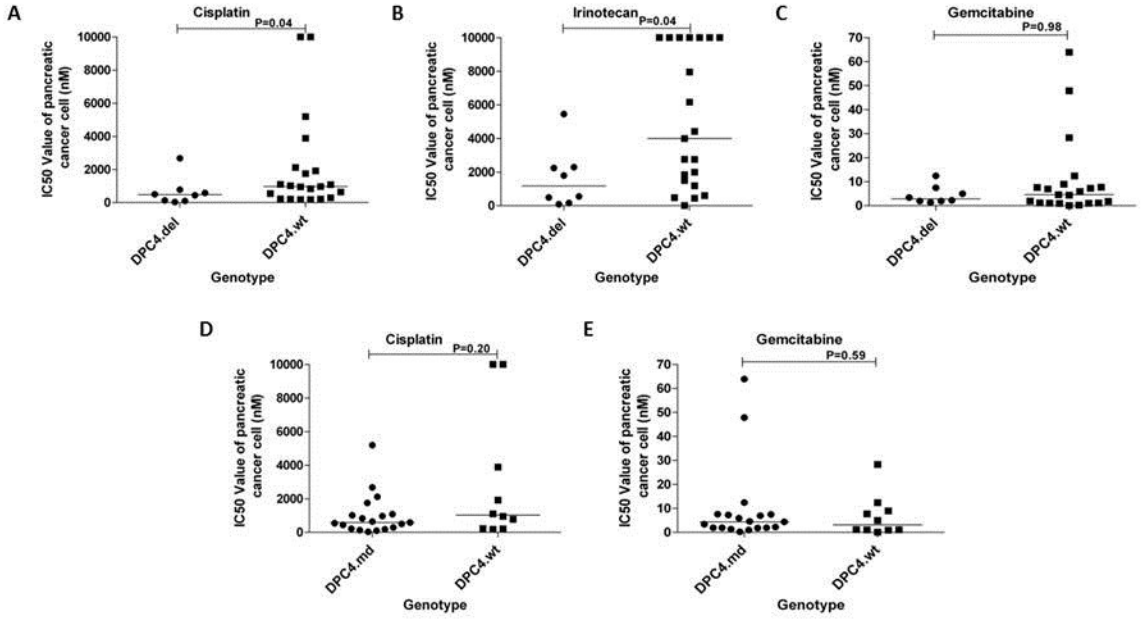

Figure 11. Correlations of drug response to DPC4 genotype.
Cytotoxic effects of cisplatin (A) and irinotecan (B) were related to DPC4/SMAD4 deletion. Gemcitabine (C, E) and cisplatin (D) (not significant) are included for the DPC4 mutation status because they have significant different drug responses in DPC4 isogenic pairs. Wilcoxon rank sum tests were used to compare differences in the median IC50 by mutation status. "md" indicates either mutation or deletion and the "wt" indicates wildtype.

Table 7. Correlation analysis between chemosensitivity and somatic mutations alone, homozygous deletion alone, or both in pancreatic cancer cells identified in the discovery and prevalence screen

Drug	Genotype	M+	M-	M+: Median (Range)	M-: Median (Range)	P value	Fold between medians
Irinotecan	SMAD4.del	8	21	1171.10 (89.72, 5454.00)	3999.00 (10.09, 10000.00)	0.040	0.29
	SMAD4.md	19	10	1998.00 (10.09, 10000.00)	8977.50 (444.90, 10000.00)	0.040	0.22
	TGFBR2.md	4	25	8977.50 (6164.00, 10000.00)	1998.00 (10.09, 10000.00)	0.030	4.49
Cisplatin	SMADPath.del	9	20	445.30 (39.36, 2682)	996.65 (185.10, 10000.00)	0.020	0.45
	SMAD4.del	8	21	476.30 (39.36, 2682)	970.30 (185.10, 10000.00)	0.040	0.49

* Median (range) IC50 values for drugs by mutation status (mutated/deleted: M+, not mutated/deleted: M-). Ic50 values were truncated at 10000. P values are for Wilcoxon rank sum tests for differences in the median IC50 by mutation status. Gene names followed by "del" indicates a deletion; "md" indicates either a mutation or deletion. SMADPath includes SMAD2, SMAD4, TGFBR2, and TGFBR3.

Table 8. Logistic regression analysis for drug response to genetic status

Drug	Genotype	M+ R+	M+ R-	M- R+	M- R-	Odds Ratio	95% CI	Exact p
Irinotecan	SMAD4.md	17	2	5	5	8.62	(1.18, 63.1)	0.020
	SMAD4.del	8	0	14	7	/	/	0.020
Cisplatin	SMAD4.del	7	1	8	13	11.93	(1.19, 119.22)	0.010
	SMADPath.md	14	9	1	5	7.56	(0.75, 76.2)	0.050

* Number of cell lines, by gene status (mutated/deleted: M+, not mutated/deleted: M-) and drug response (responder: R+, non-responder: R-). Gene names followed by "del" indicates a deletion; "md" indicates either a mutation or deletion. Odds ratios are from logistic regression models for drug response as a function of mutation status. A drug response was defined as an IC50 value below the median. P values are for Fisher's exact test. A model was run for each gene/drug combination. SMADPath includes SMAD3, SMAD4, TGFBR2, and TGFBR3. "/" indicates odds ratio and 95% CI that couldn't be calculated because one element is zero.

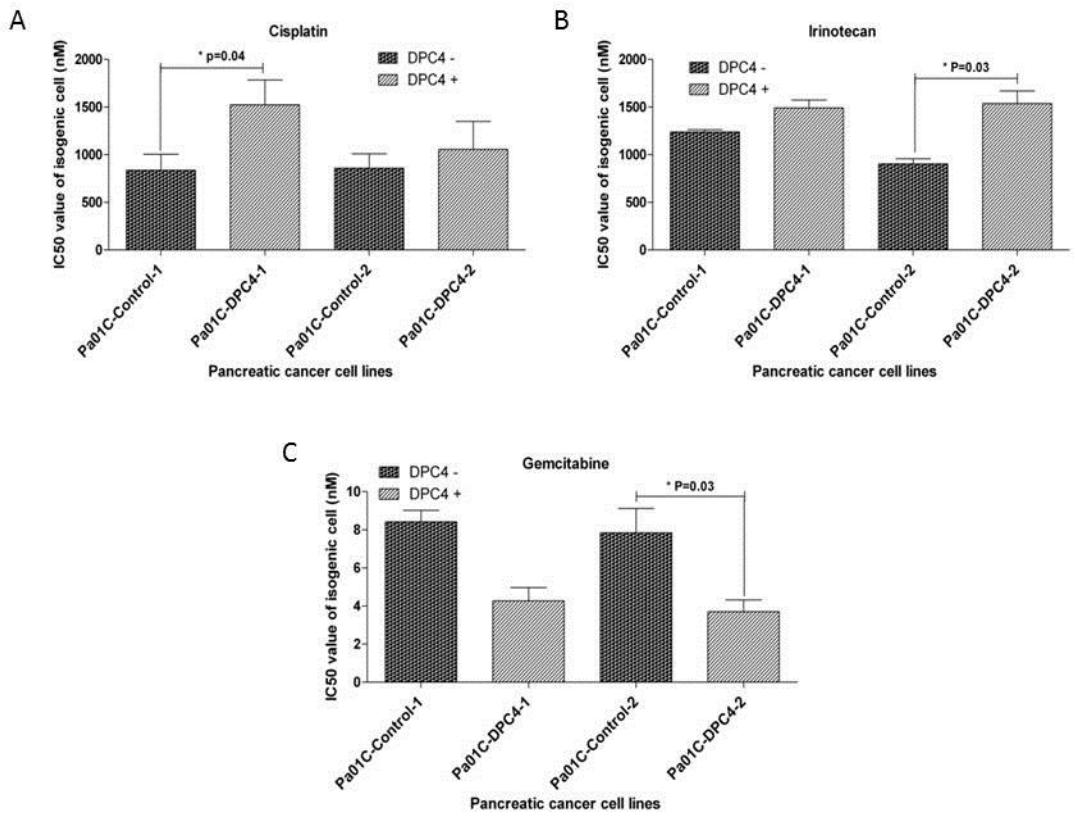

Figure 12. Responses of isogenic cancer cell lines to anticancer drugs.
Two pairs of isogenic pancreatic cancer cell lines for DPC4/SMAD4 were tested with cisplatin (A), irinotecan (B) and gemcitabine (C). Results for Pa01C isogenic cells are shown, as they demonstrate higher TGFβ functional complementation. Differences of IC50 value for isogenic pairs were compared with paired t-test analysis and significant P values indicated. IC50s were calculated for each experiment using 6 replicates per dose, and three separate experiments were used to calculate the mean IC50 for each drug and cell line combination (18 replicate wells totally per dose).

Table 9. IC50s of DPC4/SMAD4 isogenic cells tested with Cisplatin, Irinotecan, and Gemcitabine

Drug	Isogenic cell lines	Genotype of DPC4	TGFB pathway activity	IC50 (nM)	Fold between means	P
Cisplatin	Pa01C-DPC4-1	+	4.06	1524.00± 259.90	1.82	0.040
	Pa01C-Control-1	-	0.86	837.70± 166.50		
	Pa01C-DPC4-2	+	3.35	1055.00± 294.50	1.23	0.307
	Pa01C-Control-2	-	1.24	858.70± 150.70		
	Pa02C-DPC4-1	+	1.68	601.40± 47.17	1.15	0.060
	Pa02C-Control-1	-	1.11	523.00± 29.69		
	Pa02C-DPC4-2	+	1.46	809.30± 55.56	1.27	0.028
	Pa02C-Control-2	-	1.12	636.90± 75.43		
Irinotecan	Pa01C-DPC4-1	+	4.06	1491.00± 83.81	1.20	0.077
	Pa01C-Control-1	-	0.86	1239.00± 24.11		
	Pa01C-DPC4-2	+	3.35	1538.00± 131.6	1.70	0.030
	Pa01C-Control-2	-	1.24	903.60± 54.18		
	Pa02C-DPC4-1	+	1.68	1787.00± 134.30	1.40	0.080
	Pa02C-Control-1	-	1.11	1274.00± 112.40		
	Pa02C-DPC4-2	+	1.46	2393.00± 98.52	1.37	0.044
	Pa02C-Control-2	-	1.12	1745.00± 48.01		
Gemcitabine	Pa01C-DPC4-1	+	4.06	4.27± 0.70	0.51	0.058
	Pa01C-Control-1	-	0.86	8.42± 0.60		
	Pa01C-DPC4-2	+	3.35	3.70± 0.62	0.47	0.033
	Pa01C-Control-2	-	1.24	7.85± 1.27		
	Pa02C-DPC4-1	+	1.68	5.75±	0.56	0.055

			0.17		
Pa02C-Control-1	-	1.11	6.77±		
			0.14		
Pa02C-DPC4-2	+	1.46	4.73±	0.58	0.186
			0.69		
Pa02C-Control-2	-	1.12	8.14±		
			1.27		

* Differences of IC50 value for isogenic pairs were compared with paired t-test analysis.

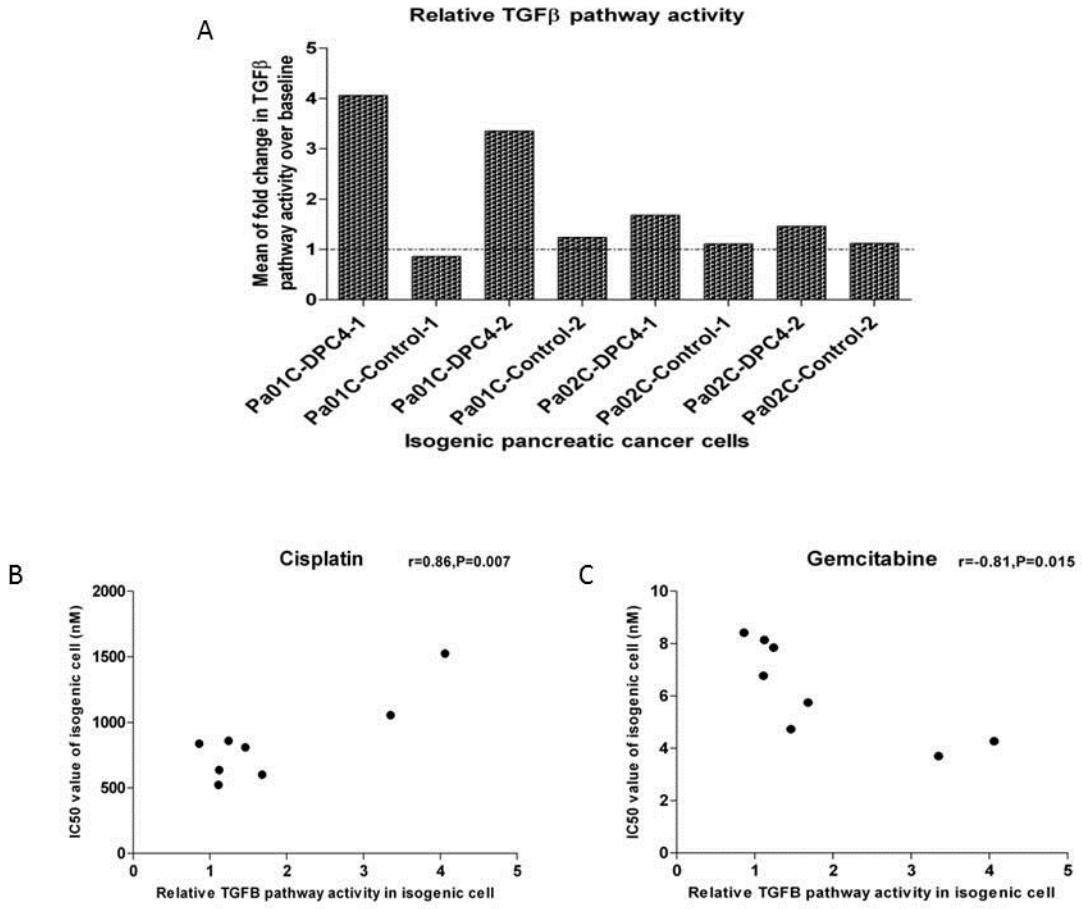

Figure 13. Association of drug response to TGF-β pathway activity.
TGF-β pathway activities were tested for DPC4 isogenic pairs using luciferase assay (A).Correlations between IC50s of cisplatin (B) or gemcitabine (C) and TGF-β pathway activity of DPC4 isogenic cells were performed by Spearman analysis.

Table 10. Correlation analysis between IC50s of Cisplatin, Irinotecan, and Gemcitabine and TGFB pathway activity of DPC4 isogenic cells

Drug	Correlation r	95% confidence interval	P value
Cisplatin	0.86	0.39 to 0.97	0.007
Irinotecan	0.07	-0.67 to 0.74	0.067
Gemcitabine	-0.81	-0.96 to -0.25	0.015

* Correlations analysis was performed by Spearman method.

Table 11. Combination indices (CI) for cisplatin+irinotecan combination in DPC4 isogenic cell lines

Cell Line	Combo	Constant Ratio	Time Schedule	CI (ID50)	CI (ID75)
Pa01C-DPC4-1	Cisplatin + Irinotecan	1:1	Simultaneously	0.66	1.04
Pa01C-Control-1	Cisplatin + Irinotecan	1:1	Simultaneously	1.31	1.73
Pa01C-DPC4-2	Cisplatin + Irinotecan	1:1	Simultaneously	0.73	0.93
Pa01C-Control-2	Cisplatin + Irinotecan	1:1	Simultaneously	4.38	1.91

* CI: synergism if <1.0, antagonism if >1.0, and additivity if 1.0.

CHAPTER 4: THE SPECTRUM OF TGF-β AND BMP SIGNALING IN PANCREATIC CANCER

Introduction

As described in Chapter 1, one of the major genetic alterations in pancreatic cancers is the loss of *SMAD4*, either through mutation or homozygous deletion (21,22,33). Of the four major driver genes classified in pancreatic cancer, *SMAD4* has been one of particular interest due to its prognostic significance: cancers harboring *SMAD4* mutations and deletions metastasize to a higher degree and cause a shortened survival compared to cancers with intact SMAD4 (42,93,94,108).

As would be expected, the TGF-β and BMP pathways, which require SMAD4 to function (36), were recently identified as being two of the most commonly altered pathways in pancreatic cancer (21). Mutations in major components of both pathways were detected in two large-scale sequencing efforts of pancreatic cancers (21,22). While mutations specific to both pathways were detected, each patient had a maximum of one TGF-β superfamily aberration. Still, there was a preponderance of mutations in SMAD4 in these cohorts; because of its role in both TGF-β and BMP responsiveness, it is difficult to glean information regarding which particular pathway's loss is selected for during pancreatic cancer evolution.

Given that SMAD4 is necessary for the full activation of canonical TGF-β and BMP signaling, we investigated these pathways in greater detail in pancreatic cancer cell lines that were previously identified as having all pathway components intact either through directed or whole-exome sequencing efforts (21,38). Herein, we investigate the nature of TGF-β and BMP signaling in tumors retaining all pathway components and

speculate to non-genetic mechanisms by which these pathways are controlled in pancreatic cancer.

Materials and Methods

Cell culture. All cell lines were maintained in Dulbecco's Modified Eagle Medium containing 1 g/L glucose (Gibco, Carlsbad, CA), 10% Fetal Bovine Serum (Gemini Bio-Products, West Sacramento, CA), 1% Penicillin/Streptomycin (Quality Biological, Gaithersburg, MD), and 5 μg/mL Plasmocin (Invivogen, San Diego, CA). HPNE and HPNE-KRAS cells were obtained from Dr. Michel M. Ouelette (152,153). All other cell lines used herein were purchased from the American Type Culture Collection (Capan2, MiaPaCa2, Panc1, and Su.86.86) or were established by our laboratory (Pa01, Pa02, Pa03, and Pa04).

Cell transfection. All transfections were performed using the Attractene lipofection system (Qiagen, Valencia, CA).

Luciferase assays. Cells were transiently co-transfected with a construct containing a firefly luciferase reporter under the control of a normal (p6SBE) or mutant Smad-binding element (pm6SBE) to measure TGF-β response or a normal (ID1 WT4F luc) or mutant (ID1 mutB4F) ID1 promoter to measure BMP response in addition to a construct containing a *Renilla* luciferase reporter (129,154). 0.01 mg/mL human TGF-β1 (Sigma, St. Louis, MO) was added to the cell medium approximately five hours after transfection, while 300 ng/mL human BMP-2 (eBioscience, San Diego, CA) was added to cell medium approximately 24 hours after transfection (154). Luciferase activity was measured 48 hours after transfection using the Dual Luciferase Reporter Assay System (Promega, Madison, WI) and a Wallac 1420 plate reader (Perkin Elmer, Waltham, MA) and normalized to *Renilla* luciferase activity.

Overexpression of KRAS. HPNE cells were transfected with a construct containing a firefly luciferase reporter under the control of a normal (p6SBE) or mutant Smad-binding element (pm6SBE) in addition to a construct containing a *Renilla* luciferase reporter (129,154); simultaneously, the cells were transfected with either an empty pBabe vector or pBabe-KRas12V (Addgene plasmid 12544) (155). 0.01 mg/mL human TGF-β1 (Sigma, St. Louis, MO) was added to the cell medium approximately five hours after transfection. Luciferase activity was measured 48 hours after transfection using the Dual Luciferase Reporter Assay System (Promega, Madison, WI) and a Wallac 1420 plate reader (Perkin Elmer, Waltham, MA) and normalized to *Renilla* luciferase activity.

Treatment with U0126. Cells were transiently co-transfected with a construct containing a firefly luciferase reporter under the control of a normal (p6SBE) or mutant Smad-binding element (pm6SBE) to measure TGF-β response or a normal (ID1 WT4F luc) or mutant (ID1 mutB4F) ID1 promoter to measure BMP response in addition to a construct containing a *Renilla* luciferase reporter (129,154). 10 μM U0126 (Cell Signaling, Danvers, MA) or an equivalent volume of DMSO was added to cells along with 0.01 mg/mL human TGF-β1 (Sigma, St. Louis, MO) or 300 ng/mL human BMP-2 (eBioscience, San Diego, CA). After six hours, luciferase assays were performed as above.

Western blotting. Cells were lysed in 1X RIPA buffer containing a cocktail of protease inhibitors. Protein concentration was determined by bicinchoninic acid protein assay (Pierce, Rockford IL), and thirty nanograms of total protein was loaded onto a 4-12% Bis-Tris polyacrylamide gel and transferred onto a nitrocellulose membrane. Antibody

conditions are listed in Table 13. Signal was detected using the ECL-Plus kit (GE Healthcare, Piscataway, NJ).

Patient samples. Patient samples were obtained from consenting patients undergoing surgical removal of pancreatic cancers at Johns Hopkins Hospital and from patients enrolled in the GICRMDP at autopsy.

Immunohistochemistry. Immunolabeling was performed using a 1:500 dilution of anti-SMAD6 antibody (Invitrogen, Carlsbad, CA) using standard methods. Cancer tissue was given an intensity score between 0-3 with consensus by two authors viewing the slides simultaneously at a two-headed microscope, with scores of 2 or greater considered "high."

RNA isolation and quantitative real-time PCR. RNA was extracted from cell lines using the RNEasy Mini Kit (Qiagen, Valencia, CA), and five hundred nanograms of RNA was converted to cDNA using the Thermoscript RT-PCR system (Invitrogen, Carlsbad, CA) and diluted ten-fold (twenty-fold for *β-actin*). Quantitative real-time PCR was performed using an Applied Biosystems 7300 Real-Time PCR System (Carlsbad, CA) and the Platinum SYBR Green qPCR Supermix-UDG (Invitrogen, Carlsbad, CA). Data were analyzed by the ΔΔCt method with normalization to *β-Actin*. Primer sequences are listed in Table 12.

Treatment of cells with MG132 and western blotting. Cells were grown to 80% confluence in 10 cm dishes and treated with 10 μM MG132 (Sigma, St. Louis, MO) or an equal volume of DMSO overnight. Cells were subsequently lysed and immunoblotting was performed as above.

Results

Baseline levels of TGF-β and BMP signaling in pancreatic cancer.

In order to obtain a better understanding of the degree to which the TGF-β and BMP signaling pathways are active in pancreatic cancer, we obtained six cell lines in which all components of these two pathways are genetically intact. Four of these cell lines (Capan2, MiaPaCa2, Panc1, and Su.86.86) underwent directed sequencing of SMAD and TGF-β receptor genes (38); two additional cell lines (Pa03 and Pa04) were included in a cohort for whole-exome sequencing (21). The degree to which the TGF-β and BMP pathways are active in these cell lines was measured using luciferase reporters specific for each network. To measure TGF-β activity, we obtained the p6SBE and pm6SBE luciferase reporters, which comprise six copies of the wild-type and mutant SMAD-binding element, respectively (129). To measure BMP activity, we obtained the ID1 WT4F and ID1 mutB4F luciferase reporters, which are composed of four copies of wild-type and mutant ID1 promoters, respectively (154); ID1 is the classical read-out for BMP activity (156).

Upon performing the luciferase screen, we observed a striking trend: despite the genetically-intact TGF-β and BMP pathways of these cell lines, the degree to which these pathways were activated more closely resembled cell lines with a homozygous SMAD4 deletion than the non-cancerous pancreas cell line, HPNE (Figure 14). This phenomenon was particularly noteworthy for TGF-β, although BMP signaling was also highly variable among the six cell lines.

The Impact of Oncogenic RAS on TGF-β and BMP Signaling in Pancreatic Cancer

Given the surprisingly low levels of TGF-β and BMP signaling in pancreatic cancer cell lines wild-type for the components of these pathways, we hypothesized that these networks may be inhibited by another mechanism. Previous studies by Massagué and colleagues had determined that the presence of oncogenic mutations in *KRAS* interfered with the ability of colorectal cancer cells to respond to TGF-β ligand (157,158). It was determined that the MEK-ERK cascade, activated downstream of RAS, causes phosphorylation of the linker regions of both TGF-β- and BMP-associated R-SMADs, preventing nuclear import of these critical signaling mediators (23,157-159). Given the nearly ubiquitous presence of constitutively active KRAS in pancreatic cancer (20-22), we sought to determine whether these mutations were contributing to the dampened response to TGF-β and BMP in the cell lines used herein.

In order to ascertain whether hyperactive KRAS affects pancreatic cancer cells' response to the TGF-β superfamily, HPNE (152) and HPNE-KRAS (153) cells were analyzed for TGF-β and BMP response. HPNE-KRAS cells express a common variant of this oncogene in pancreatic cancer, G12D (153). Contrary to our expectations, the presence of $KRAS^{G12D}$ significantly impacted neither TGF-β nor BMP response levels (Figure 15A). In addition, we repeated the TGF-β specific assay after transfecting HPNE cells with a different oncogenic KRAS variant, G12V (155). This variant also failed to significantly inhibit TGF-β signaling (Figure 15B). However, while non-significant, KRAS appears to slightly activate TGF-β signaling in these cells (Figure 15). These data imply that the dampening of TGF-β and BMP signaling in pancreatic cancer cells is due to a factor other than RAS. In order to verify this possibility, we repeated the TGF-β and

BMP reporter assays in the presence or absence of U0126, a pharmaceutical MEK inhibitor, to inhibit the ERK-mediated phosphorylation of R-SMADs (160). HPNE, MiaPaCa2, and Panc1 cells were treated with U0126 prior to performing the TGF-β and BMP reporter assays. Should ERK be phosphorylating the linker region of R-SMADs to inhibit downstream TGF-β and BMP responses, we would expect an increase in the activity of these pathways upon treatment with U0126. While there was a small increase in TGF-β activity in MiaPaCa2 ($p=0.04$), there was, otherwise, no significant change in the level of TGF-β (Figure 16A) or BMP signaling (Figure 16B) in these cell lines. We conclude, therefore, that the activating mutations in *KRAS* that are observed in the majority of pancreatic cancers are not substantially impacting the ability of the cancer cells to respond to TGF-β and BMP ligands.

The Impact of Endogenous TGF-β and BMP Antagonists on TGF-β and BMP Signaling in Pancreatic Cancer

If RAS is not inhibiting TGF-β and BMP responses in pancreatic cancer cells, there likely exists another factor that is impairing these pathways' activity. An intriguing notion is that an antagonist of the TGF-β and BMP pathways is overexpressed in SMAD4-intact cases to compensate for the continued activation of these pathways. To investigate this possibility, we performed western blotting against several known inhibitors of TGF-β and BMP signaling to assess their expression in cell lines wild-type for TGF-β and BMP pathway components. Examples of these immunoblots are shown in Figure 17. While we noted expression of several inhibitors across our panel, we were

particularly struck by the stark differential expression of SMAD6, an inhibitor of both TGF-β and BMP signaling, among these cell lines (74,75).

Recognizing that cell lines do not always provide an accurate representation of the biology of pancreatic cancers *in vivo*, we sought to verify the expression of SMAD6 within pancreatic cancer samples. We performed immunohistochemistry for SMAD6 on primary pancreatic cancers from 180 patients (from surgical resection and rapid autopsy) and observed a similar disparity in SMAD6 expression (Figure 18). Out of the cases examined, 70.6% displayed strong SMAD6 expression, whereas the remainder showed barely detectable levels of the protein. Therefore, we conclude that SMAD6 expression is extremely variable among pancreatic cancer patients.

Analysis of SMAD6 regulation.

Given our observation that SMAD6 expression is elevated in the majority of pancreatic cancers, we considered the possibility of an activating mutation in this gene. Previously, a directed sequencing study directed at all SMAD genes did not detect *SMAD6* mutations in twelve pancreatic cancer cell lines, nor in other tumor types (161). Since, there have been two large-scale sequencing efforts in pancreatic cancer, compiling genetic data on a combined 123 patients; not a single *SMAD6* mutation was identified in these cohorts (21,22). As such, we surmised that *SMAD6* mutation is unlikely the cause of this gene's differential regulation. Therefore, we sought to determine the epigenetic mechanism through which SMAD6 expression is regulated in pancreatic cancer.

Given that SMAD6 is unlikely to be activated by mutation in pancreatic cancer (21,22,161), another logical possibility is that the level of its transcription is being aberrantly regulated. There are several factors known to induce transcription of *SMAD6*, including stimulation with TGF-β1, activin, BMP-2, BMP-7, and EGF (162,163). These data suggest that *SMAD6* is activated in response to TGF-β and BMP as endogenous negative feedback; consistent with this notion, *SMAD6* was not induced by TGF-β or BMP ligands in cells lacking SMAD4 (162).

SMAD6 is transcribed at a baseline level in several normal human tissues, including pancreas (74); however, we wished to determine if this expression was dysregulated in pancreatic cancer. Kleeff *et al.* previously demonstrated an upregulation of *SMAD6* mRNA in pancreatic cancer cells compared to normal ductal epithelial cells, but were unable to correlate levels of *SMAD6* transcript to protein (110). In order to determine if the varied SMAD6 levels observed previously are the result of differential transcription of this locus in pancreatic cancer, we extracted RNA from Capan2, MiaPaCa2, Panc1, Su.86.86, Pa03, and Pa04 cells and performed quantitative real-time PCR to measure the levels of *SMAD6* RNA. *SMAD6* transcript was detected in all cell lines examined, in agreement with prior studies (74,161,162). However, the levels of *SMAD6* transcript did not correspond to its protein levels, indicating that control of *SMAD6* transcription is unlikely the cause of its differential protein expression (Figure 17, Figure 19).

Because SMAD6 does not appear to be regulated at the DNA or RNA level, we concluded that regulation via a post-transcriptional mechanism was likely. To assess this possibility, we treated the cell lines negative for SMAD6 protein expression (Capan2,

Su.86.86, Pa03, and Pa04) with MG132, a peptide aldehyde that functions to inhibit the proteasome (164). Upon treatment with the proteasome inhibitor, SMAD6 protein levels were restored in each of these four cell lines (Figure 20). The difference in size between the restored SMAD6 proteins in these cell lines and the endogenous SMAD6 in MiaPaCa2 was consistent with SMAD6 being polyubiquitinated. As such, we are able to conclude that aberrant degradation of SMAD6 is, at least, partially responsible for its differential expression in pancreatic cancer.

Discussion

Through analysis of genetic mutations discovered through whole-exome sequencing, the TGF-β and BMP pathways were identified as among those most commonly dysregulated in pancreatic cancers (21). Herein, we have determined that, even in cases that would be classified as having unaltered TGF-β and BMP networks by genetic analyses, TGF-β and BMP signaling is distorted (Figure 14). These results confirm those of Simeone *et al.*, who noted a lack of TGF-β activation in the MiaPaCa2 and Panc1 cell lines (148). Furthermore, Dai and colleagues showed that SMAD4 restoration into BxPC3 cells did not rescue TGF-β response, but did in a breast cancer cell line, MDA-MB-468 (149). This finding is in agreement with our observation that SMAD4 complementation has little effect on the TGF-β activity of Pa01 and BxPC3 cells (Figure 2B).

That the degrees of TGF-β and BMP activity do not correspond to the mutational profile determined previously (21,38) suggests that these pathways are being regulated both genetically, via mutation or deletion of *SMAD4* and other pathway components, and epigenetically in pancreatic cancer. Given the near universal dampening of signaling – particularly TGF-β (Figure 14) – we considered the likelihood that constitutively active KRAS was the causative factor for this phenomenon (157-159). Previous studies have implicated MEK-ERK activity downstream of RAS in the inhibition of R-SMAD nuclear import (157-159). Given the propensity of *KRAS* mutations in pancreatic cancer (nearly all patients have a mutation in this gene) (20-22), we considered the likelihood that RAS signaling was impinging on the SMAD pathways in our model. However, neither common *KRAS* variants nor MEK-ERK blockade significantly impacted the degree of

SMAD signaling in normal and cancerous pancreatic cell lines (Figure 15, Figure 16). The lack of TGF-β response in SMAD4-complemented BxPC3 cells supports these results: BxPC3 retains wild-type *KRAS*, so would not be susceptible to KRAS-mediated TGF-β antagonism (149). The studies that associated oncogenic RAS with TGF-β and BMP inhibition were performed in other cell models (157-159). The possibility exists that there are cell-type reasons for this discrepancy.

After eliminating oncogenic RAS as a mechanism of TGF-β and BMP impediment in pancreatic cancer cells, we considered the option that an endogenous antagonist was overexpressed to compensate for the presence of wild-type pathway members. There are many known antagonists of TGF-β and BMP signaling. These factors impact the pathways at varying levels from the membrane to the nucleus. There are several extracellular ligands that prevent the binding of TGF-β and BMP ligands to their receptors, including NOGGIN, CHORDIN, and FOLLISTATIN, among others (reviewed in (165)). BAMBI is a kinase-dead version of type I TGF-β receptors (79). There are several inhibitors that prevent phosphorylation of R-SMADs by the receptors, including SMAD6, STRAP, and SMAD7 (72,74,78). Many proteins have been identified that contribute to the ubiquitin-mediated degradation of pathway components, such as SMAD6, SMAD7, PIN1, SMURF1, SMURF2, NEDD4-2, WWP1, CHIP, JAB1, and ROC1 (62,64-70,73,77). Lastly, there are nuclear proteins that complex with SMADs to form a repressive, rather than an active, complex on promoter regions: SKI, SNO, SMAD6, and PARP-1 (76,80-82,84,85).

We sought to determine whether any of these inhibitors could factor into TGF-β and BMP inhibition in pancreatic cancer. Of these factors, there are several that have

been studied in the context of pancreatic cancer. SMAD7 expression leads to a more aggressive phenotype in pancreatic cancer cell lines, while overexpression of SMAD7 in mouse pancreata enhances the formation of PanIN lesions (166,167). Similarly, SMURF1 was found to be amplified in a small subset of pancreatic cancers, leading to increased invasion and anchorage-independent growth (168). However, increased expression of SKI correlated with reduced levels of invasion and metastasis (169). Therefore, much like TGF-β and BMP signaling, in general, inhibitors of these pathways exert varied effects on pancreatic cancer cells.

Through immunoblotting for several of these antagonists in our genetically wild-type cell line panel, we have identified an intriguing differential expression pattern of one such inhibitor, SMAD6 (Figure 17). We were subsequently able to verify this differential expression in primary pancreatic cancers (Figure 18). SMAD6 has previously been shown to be overexpressed at the RNA level in a subset of pancreatic cancers (110); here, we have shown differential expression of SMAD6 protein for the first time in this cancer type. The previously described functions of SMAD6 are particularly intriguing in regards to its overexpression in pancreatic cancer. Given that SMAD6 can inhibit both TGF-β and BMP signaling (74,75), it is intriguing to speculate that SMAD6 may phenocopy SMAD4 loss, since SMAD4 is essential for both pathways (36).

Cancer is frequently thought of as a disease that is fueled by the accumulation of genetic mutations (170). However, there are multiple routes to a gain or loss of function of a protein. For example, as described in this chapter, SMAD6 expression appears to be regulated at the post-transcriptional level by aberrant ubiquitination, rather than by genetic mutation (21,22,161) or altered transcription (Figure 19, Figure 20). This notion

is furthered by the observation that ubiquitin is, indeed, one of the proteins previously found to bind SMAD6 (171).

Ubiquitination is a highly conserved biological process present across eukaryotes. Ubiquitin, itself, is a relatively small (76 amino acid) protein that is covalently attached to lysine residues on target proteins via a C-terminal glycine residue (172). The process of ubiquitination occurs in three steps. Ubiquitin proteins are activated by E1 enzymes, then transferred to a carrier protein, E2 (173). Finally, E3 catalyzes the adduction of ubiquitin onto target proteins. Polyubiquitin chains (at least four in length) that are attached to lysine residues are necessary for recognition by the proteasome (174-176).

While cancer is, generally, thought to be driven by mutations, altered ubiquitination is, by no means, a new phenomenon in the genomic regulation of oncogenic processes. Perhaps the most common cancer protein that is controlled by ubiquitination is TP53. MDM2 is a ubiquitin ligase that controls TP53 stability (177,178). Given its critical role in regulating TP53 function, it is not surprising that MDM2 amplification (leading to increased TP53 degradation) has been observed in several cancer types (179). Additional ubiquitin-mediated control of TP53 occurs in cervical cancer. The E6 protein of the human papilloma virus mediates ubiquitin-mediated destruction of TP53 by the E6-AP ubiquitin ligase (180). Not only are tumor suppressors, such as TP53, lost through over-ubiquitination, oncogenes can be activated by loss of ubiquitination, as well. For example, the epidermal growth factor receptor (EGFR), when mutated in glioblastoma, is unable to undergo degradation mediated by CBL ubiquitin ligases (181). Additional ubiquitin-ligases that have been shown to have altered function in cancers include BRCA1 and VHL (reviewed in (182)).

While we have determined that the mechanism for the differential expression of SMAD6 is, in part, altered degradation, the causal factor for this phenomenon has yet to be identified. Recently, Zhang et al. performed a screen to identify binding partners of SMAD6 (171). Of those identified, there are many candidates for ubiquitin-mediated regulation. Of the binding partners of SMAD6, several are known to function as part of the ubiquitin conjugating cascade. These proteins include the following E2 and E3 ligases: SMURF2, ITCH, WWP1, WWP2, UBE2O, UBR4, UBR5 (171). It is possible that the function of one or more of these SMAD6 binding partners is altered in pancreatic cancers, leading to the dysregulation of SMAD6 expression. Furthermore, it should be highlighted that, like most biological processes, ubiquitination is reversible. Additional binding partners of SMAD6 include several ubiquitin specific peptidases (USP7, USP9X, USP11, and USP34) (171). This class of proteins is responsible for the rescue of proteins from proteolytic degradation (reviewed in (183)). It is worth noting that several of these ubiquitin-modulating factors – both ubiquitin ligases and de-ubiquitinating enzymes – have been identified as being mutated in pancreatic cancer (21,22); however, these mutations are quite rare. Therefore, it is plausible that there are a multitude of mechanisms through which SMAD6 ubiquitination is altered in pancreatic cancer patients.

In Chapter 2, we were able to associate the loss of SMAD4 with an increased rate of proliferation, migration, and invasion, while previous work has demonstrated an association between SMAD4 loss, elevated metastasis, and poor prognosis (42,93,94). It is tempting to assume that SMAD6 elevation phenocopies SMAD4 loss in pancreatic cancer; however, TGF-β and BMP pathway inhibitors appear to have pleiotropic effects

on pancreatic cancer, based on prior studies (110,166-169). The relationship between SMAD6 and pancreatic cancer will be further addressed in the next chapter.

Table 12. Primer sequences used for real-time PCR.

Primer Name	Primer Sequence
SMAD6 Forward	5-ACCAACTCCCTCATCACTGC-3
SMAD6 Reverse	5- CTGCCCTGAGGTAGGTCGTA-3
β-Actin Forward	5-GACCCAGATCATGTTTGAGAC-3
β-Actin Reverse	5-GATGGGCACAGTGTGGGTGAC-3

Table 13. Antibodies used for immunoblotting.

Antibody	Company	Dilution
SMAD6	Imgenex (San Diego, CA)	1:500
PARP-1	Cell Signaling (Danvers, MA)	1:1000
SKI	Santa Cruz Biotechnology (Santa Cruz, CA)	1:500
SMAD7	Abcam (Cambridge, MA)	1:1000
STRAP	Sigma (St. Louis, MO)	1:500
Ubiquitin	Cell Signaling (Danvers, MA)	1:1000
GAPDH	Cell Signaling (Danvers, MA)	1:5000
HRP-linked anti-rabbit IgG	GE Healthcare (Piscataway, NJ)	1:5000

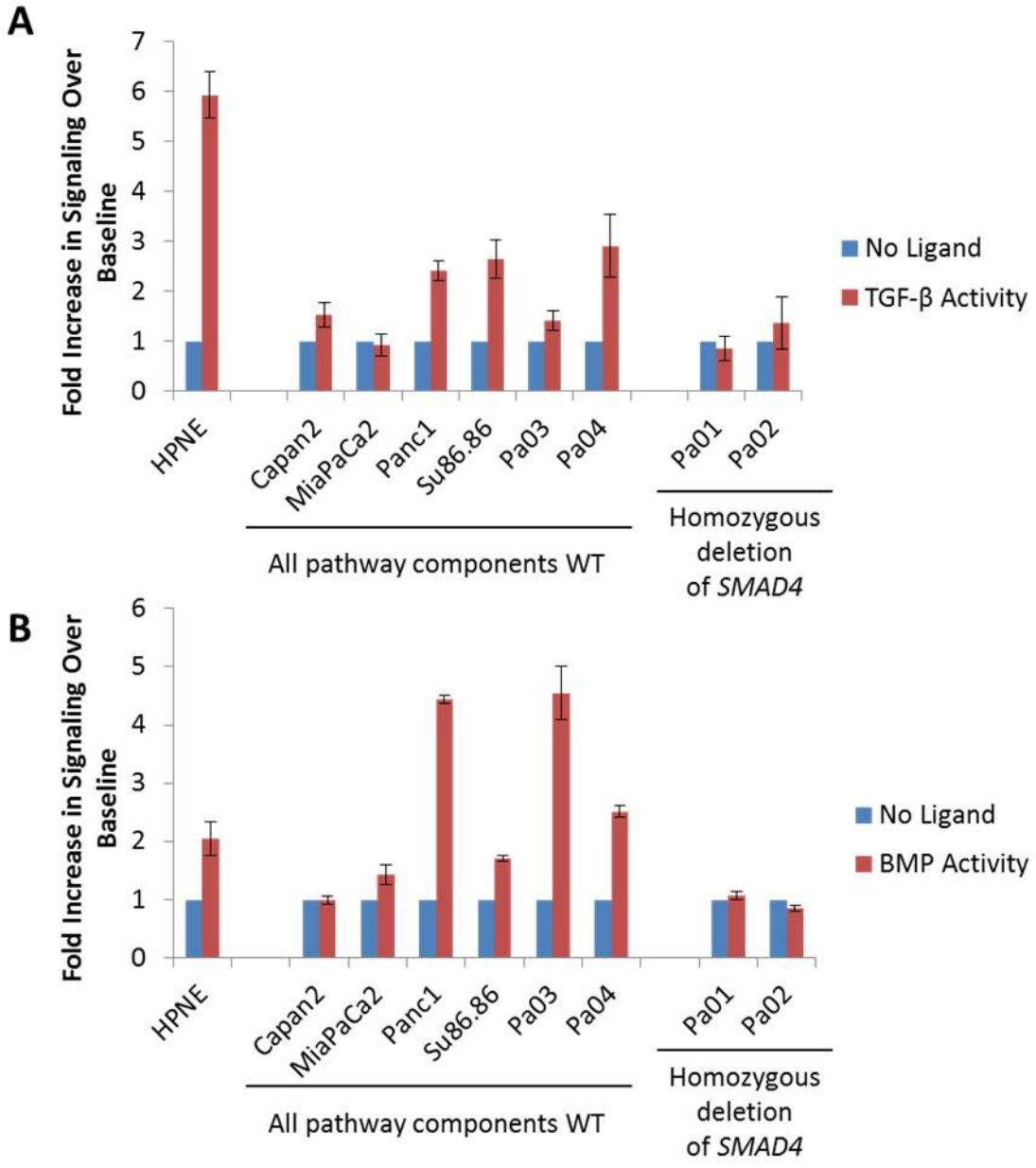

Figure 14. Luciferase screen for TGF-β and BMP response in pancreatic cancer cell lines.

Data are presented such that the wild-type to mutant promoter ratio is set to 1. TGF-β (A) and BMP (B) pathway activity is presented as the fold increase in luciferase levels after addition of ligand (e.g. SBE + TGF-β/mSBE + TGF-β). The genetic status of each cell line, determined by either directed or whole exome sequencing (21,38), is indicated. Error bars represent standard error of the mean.

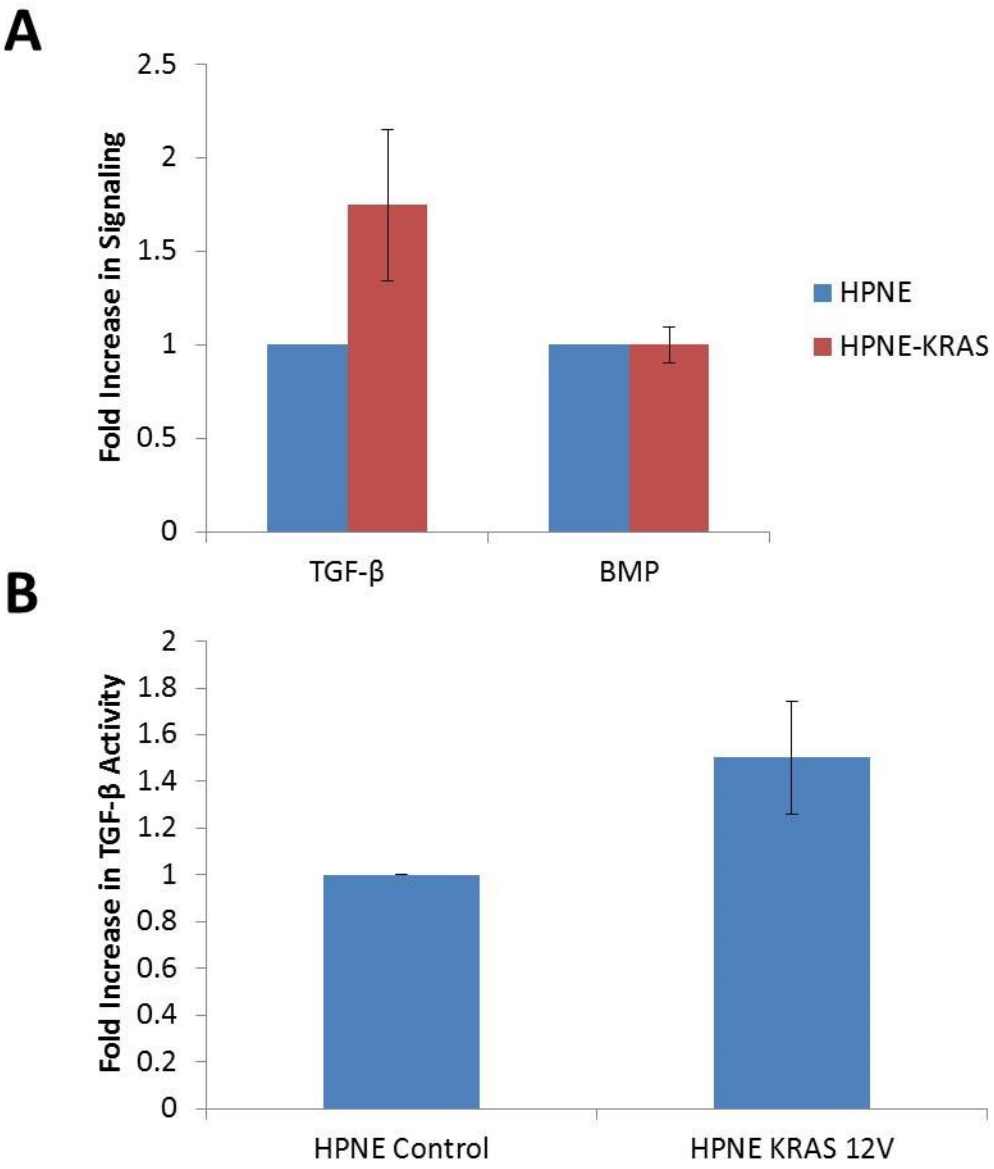

Figure 15. The effect of activated KRAS on TGF-β and BMP activity.
A) The levels of TGF-β and BMP activity were analyzed in HPNE and HPNE expressing the $KRAS^{G12D}$ mutation. B) The levels of TGF-β were analyzed in HPNE cells transiently transfected with $KRAS^{G12V}$ or an empty vector. Data are presented such that the wild-type to mutant promoter ratio is set to 1. TGF-β and BMP pathway activity is presented as the fold increase in luciferase levels after addition of ligand (e.g. SBE + TGF-β/mSBE + TGF-β). Error bars represent standard error of the mean. Significance was calculated by student's t-test.

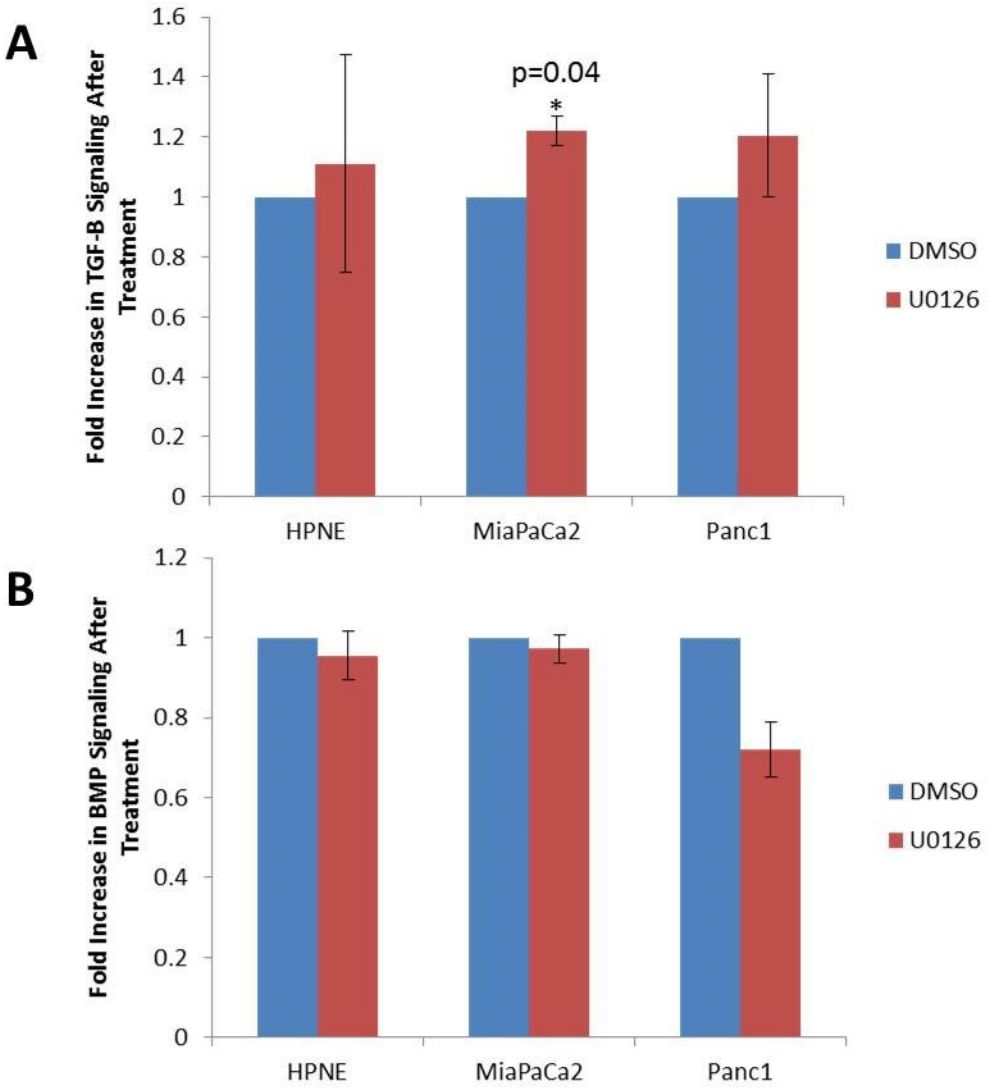

Figure 16. The effect of MEK-ERK activity on TGF-β and BMP response.
Data are presented such that the wild-type to mutant promoter ratio is set to 1. TGF-β (A) and BMP (B) pathway activity is presented as the fold increase in luciferase levels after addition of ligand (e.g. SBE + TGF-β/mSBE + TGF-β). Error bars represent standard error of the mean. Significance was calculated using student's t-test.

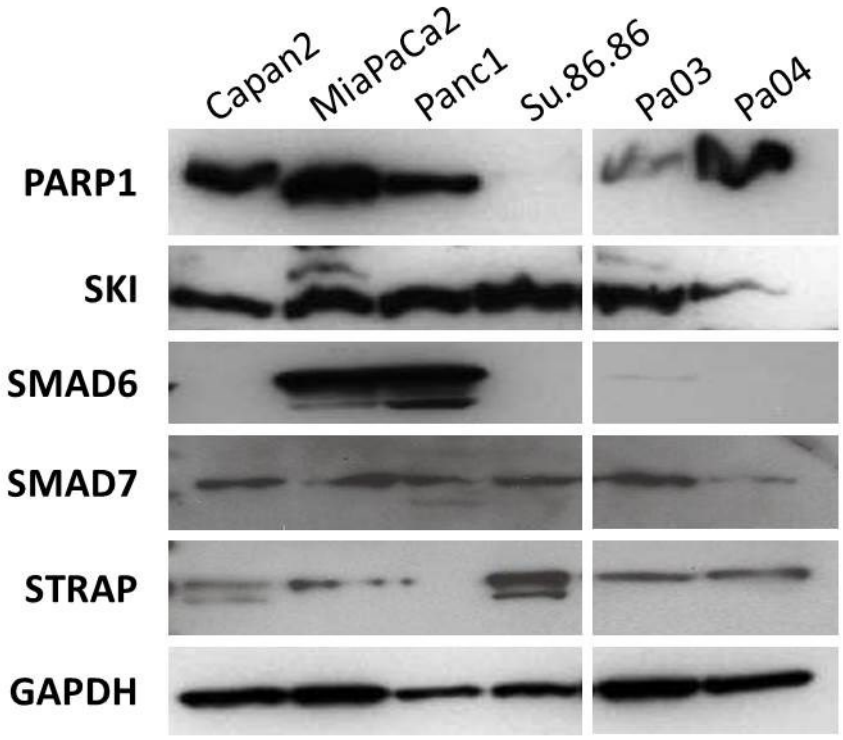

Figure 17. Expression of TGF-β and BMP antagonists in pancreatic cancer cell lines.
Expression levels of the TGF-β and BMP inhibitors PARP1, SKI, SMAD6, SMAD7, and STRAP were determined in six pancreatic cancer cell lines wild-type for all components of the TGF-β and BMP pathways and normalized to GAPDH expression.

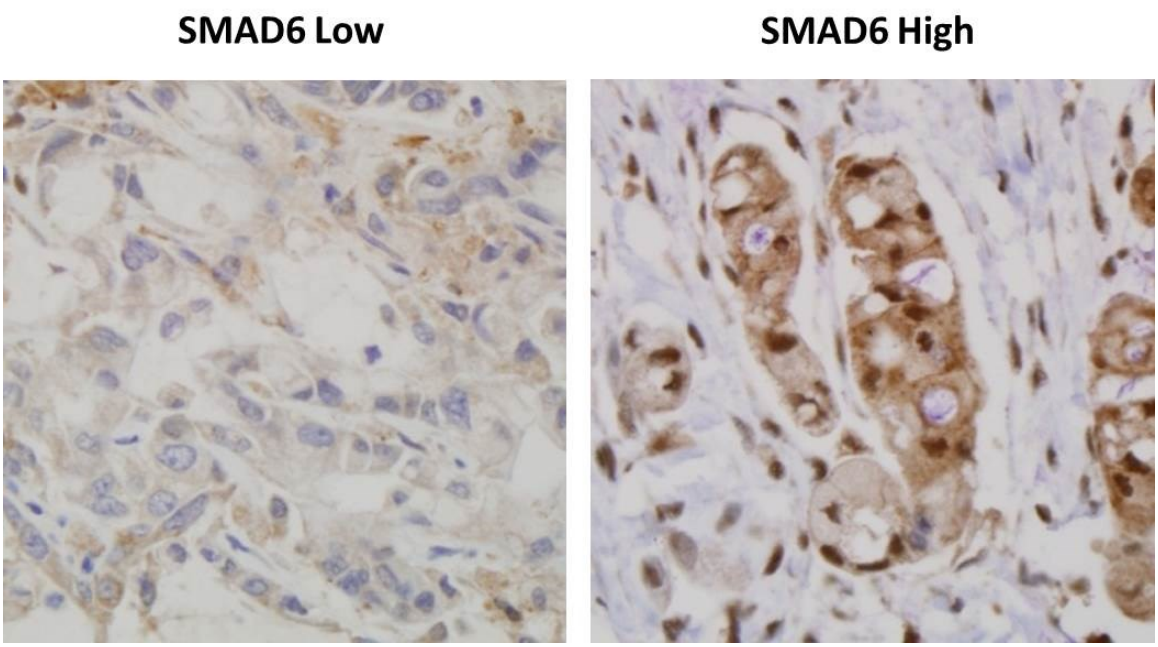

Figure 18. Expression of SMAD6 in pancreatic cancer tissues.
Examples of cancers determined to express low levels of SMAD6 (left) and high levels of SMAD6 (right) are shown.

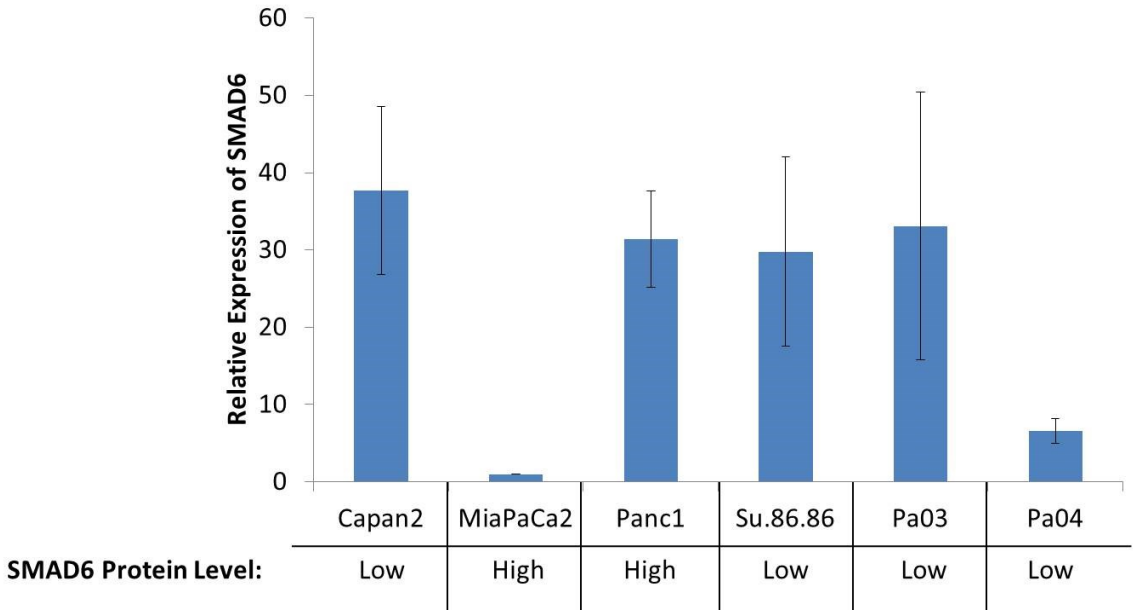

Figure 19. Relative expression of *SMAD6* transcript in pancreatic cancer cell lines. Real-time PCR for *SMAD6* expression in Capan2, MiaPaCa2, Panc1, Su.86.86, Pa03, and Pa04 cells. Levels are normalized to the expression level in MiaPaCa2. SMAD6 protein levels are also indicated (Figure 17). Error bars represent standard error of the mean.

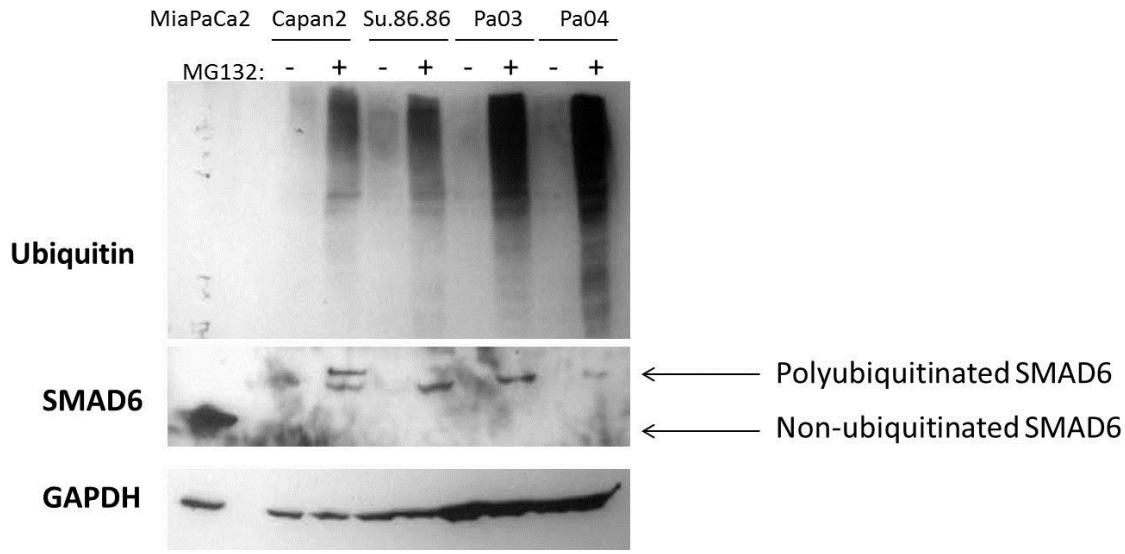

Figure 20. Proteasome inhibition rescues SMAD6 expression.
Total protein was extracted from MiaPaCa2, Capan2, Su.86.86, Pa03, and Pa04 cells in the presence or absence of the proteasome inhibitor, MG132. Ubiquitin and SMAD6 levels were determined by immunoblotting relative to GAPDH.

CHAPTER 5. THE INFLUENCE OF SMAD6 ON PANCREATIC CANCER

Introduction

As described in the preceding chapter, we have identified SMAD6, previously classified as an inhibitor of TGF-β and BMP signaling (74,75) as being differentially expressed in pancreatic cancer cell lines (Figure 17) and tissues (Figure 18). Furthermore, we have determined that SMAD6 is regulated at the protein level, through altered proteolytic degradation (Figure 20). However, it remains to be seen whether SMAD6 expression in pancreatic cancers contributes to disease progression.

It seems to make sense, given the involvement of SMAD6 in both TGF-β and BMP signaling, that overexpression of SMAD6 would phenocopy loss of SMAD4 in pancreatic cancer, in that it would lead to increased proliferation (Figure 5), migration (Figure 6A), and invasion (Figure 6B) of cancer cells, reduced survival (93,94) and increased metastasis in patients (42). However, the TGF-β and BMP pathways, overall, have less clear contributions to the development of pancreatic cancer (93,95-97,99-108). Prior studies of the link between SMAD6 and cancer cell behavior are limited. SMAD6 was associated with increased proliferation, decreased apoptosis, and a shortened survival in non-small cell lung cancer (111), while *SMAD6* expression was associated with increased anchorage-independent growth in a previous pancreatic cancer study (110). Thus, in this chapter, we seek to further define the role that SMAD6 plays – if any – in the progression of pancreatic cancer.

Materials and Methods

Cell culture. All cell lines were maintained in Dulbecco's Modified Eagle Medium containing 1 g/L glucose (Gibco, Carlsbad, CA), 10% Fetal Bovine Serum (Gemini Bio-Products, West Sacramento, CA), 1% Penicillin/Streptomycin (Quality Biological, Gaithersburg, MD), and 5 μg/mL Plasmocin (Invivogen, San Diego, CA). MiaPaCa2 and Panc1 cells were purchased from the American Type Culture Collection (Manassas, VA). Pa01, Pa03, and Pa04 cells were established by our laboratory.

Cell transfection. All transfections were performed using the Attractene lipofection system (Qiagen, Valencia, CA).

Smad6 modulation. Panc1 and MiaPaCa2 cells were transiently transfected with a scrambled shRNA construct as well as a shRNA construct designed to target SMAD6 (both purchased from Origene, Rockville, MD). Pa01, Pa03, and Pa04 cells were transiently transfected with an empty vector (pcDNA3.1(Hyg)), a SMAD6 vector (pcDNA3.1(Hyg)-SMAD6). This overexpression vector was generated through subcloning of SMAD6 cDNA purchased from Origene (Rockville, MD) into the pcDNA3.1(Hyg) vector (Invitrogen, Carlsbad, CA). SMAD6 knockdown and overexpression was verified 72 hours after transfection by immunoblotting for SMAD6.

Western blotting. Cells were lysed in 1X RIPA buffer containing a cocktail of protease inhibitors. Protein concentration was determined by bicinchoninic acid protein assay (Pierce, Rockford IL), and thirty-five nanograms of total protein was loaded onto a 4-12% Bis-Tris polyacrylamide gel and transferred onto a nitrocellulose membrane. Antibody conditions are listed in Table 14. Signal was detected using the ECL-Plus kit

(GE Healthcare, Piscataway, NJ). For nuclear and cytopasmic extracts, cellular subfractionation was performed using the NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce, Rockford, IL).

Flow cytometry. Cells were transfected to knock down SMAD6 as above. After 72 hours, cells were stained with Annexin V and propidium iodide using the Annexin V/Dead Cell Apoptosis Kit (Molecular Probes, Eugene, OR). Stained cells were analyzed by flow cytometry using a FACSCalibur flow cytometer (BD, Franklin Lakes, NJ). Data were analyzed using CellQuest Pro software (BD, Franklin Lakes, NJ).

Cell proliferation analysis. Cells were seeded in 96-well plates at a concentration of $2.5x10^4$ cells/mL and allowed to adhere overnight. Cells were transfected to modulate SMAD6 as above. 24, 48, and 72 hours after transfection, cells were treated with 10 μL of CCK8 solution (Dojindo, Rockville, MD) and incubated at 37^oC for three hours. Absorbance at 490 nm was measured using a Wallac 1420 plate reader (Perkin Elmer, Waltham, MA).

Migration and invasion analysis. Migration and invasion were measured by Boyden chamber analysis (BD Biosciences, San Jose, CA). Optimal experimental conditions were determined for each cell line. For migration, 25,000 cells (MiaPaCa2, Panc1) were plated into uncoated Boyden chambers 24 (MiaPaCa2) or 48 (Panc1) hours after transfection with a 0.5% to 10% FBS gradient and allowed to migrate for 48 (MiaPaCa2) or 24 (Panc1) hours. For invasion, 25,000 cells (MiaPaCa2, Panc1) were plated onto Boyden chambers coated with matrigel 24 hours after transfection and allowed to invade for 48 hours through a 0.5% to 10% FBS gradient (MiaPaCa2, Panc1) or 50,000 cells

were plated onto Boyden chambers 24 hours after transfection and allowed to invade for 48 hours towards 50,000 CAF35 cells (Pa03). At the endpoint of the experiment, cells having migrated or invaded were fixed and stained using Diff-Quik (Siemens, Deerfield, IL) or hematoxylin and eosin. Cells were counted in three independent fields per chamber using an inverted microscope.

Patient samples. Patient samples were obtained from consenting patients undergoing surgical removal of pancreatic cancers at Johns Hopkins Hospital and from patients enrolled in the GICRMDP at autopsy.

Immunohistochemistry. Immunolabeling was performed using the antibodies in Table 15 using standard methods. For SMAD6, cancer tissue was given an intensity score between 0-3 with consensus by two authors viewing the slides simultaneously at a two-headed microscope. Scores of 2 or greater were deemed "high." For SMAD4, cancer tissue was judged to be positive or negative.

Luciferase assays. Pa03 and Pa04 cells were transiently co-transfected with a construct containing a firefly luciferase reporter under the control of a normal (p6SBE) or mutant Smad-binding element (pm6SBE) or a normal (ID1 WT4F luc) or mutant (ID1 mutB4F) ID1 promoter in addition to a construct containing a *Renilla* luciferase reporter (129,154). 0.01 mg/mL human TGF-β1 (Sigma, St. Louis, MO) was added to the cell medium approximately five hours after transfection, while 300 ng/mL human BMP-2 (eBioscience, San Diego, CA) (154) was added to cell medium approximately 24 hours after transfection. Luciferase activity was measured 48 hours after transfection using the

Dual Luciferase Reporter Assay System (Promega, Madison, WI) and a Wallac 1420 plate reader (Perkin Elmer, Waltham, MA) and normalized to *Renilla* luciferase activity.

Chromatin immunoprecipitation. MiaPaCa2 and Pa03 cells were transiently transfected with CS2-FLAG-SMAD6 (Addgene plasmid 14961) (75) using the Attractene reagent (Qiagen, Valencia, CA) or left untransfected. Cells (transfected and untransfected) were crosslinked with formaldehyde at a final concentration of 1%. Chromatin immunoprecipitation was performed using the EZ-ChIP kit (Millipore, Billerica, MA). Conditions used for pull-down were as follows: 10 μg M2 anti-FLAG (Sigma, St. Louis, MO), 1 μg normal Mouse IgG (Millipore, Billerica, MA). ChIP results were verified using quantitative real-time PCR and calculating fold enrichment of FLAG-SMAD6 transfected samples compared to untransfected samples. Primers were designed to include the predicted SMAD6 binding site on the ID1 promoter (113).

Trichostatin A treatment. Pa03 cells were transfected to overexpress SMAD6 as above. After 48 hours, either ethanol (vehicle) or 10 ng/mL trichostatin A (TSA) was added to the cell media in the presence or absence of 200 ng/mL human BMP-2 (eBioscience, San Diego, CA) for 24 hours prior to RNA extraction for qPCR analysis. Alternatively, Pa03 and Pa04 cells were transiently co-transfected with SMAD6-overexpression constructs, a construct containing a firefly luciferase reporter under the control of a normal (ID1 WT4F luc) or mutant (ID1 mutB4F) ID1 promoter, and construct containing a *Renilla* luciferase reporter (129,154). After 48 hours, either ethanol (vehicle) or 10 ng/mL trichostatin A (TSA) was added to the cell media in the presence or absence of 300 ng/mL human BMP-2 (eBioscience, San Diego, CA). After an additional 24 hours, luciferase assays were performed as above.

BMP modulation. MiaPaCa2 cells were treated with 200 ng/mL human BMP-2 (eBioscience, San Diego, CA), while Pa03 cells were treated with 20 ng/mL anti-BMP-2/4 antibody (R&D Systems, Minneapolis, MN). Cell behavior assays were performed as above.

Statistical analysis. Statistical analyses were performed using SPSS, SISA, and Microsoft Excel.

Results

The effect of SMAD6 on pancreatic cancer cell properties.

In their seminal review and recent update, Hanahan and Weinberg describe the general properties that define cancer cells (184,185). Herein, we have sought to determine the effect of modulating SMAD6 expression on these behaviors in pancreatic cancer cells, *in vitro*.

Among the hallmarks that Hanahan and Weinberg define of cancer cells are "resisting cell death," "sustaining proliferative signaling," and "evading growth suppressors" (184,185). In order to determine whether SMAD6 causes these effects in pancreatic cells, we modulated the levels of SMAD6 in our pancreatic cancer cell lines and determined the resulting change in cell viability. We transiently knocked down SMAD6 in MiaPaCa2 and Panc1 (Figure 21A), which overexpress SMAD6 (Figure 17). In addition, we overexpressed SMAD6 in Pa03 cells (Figure 21B), which do not display endogenous SMAD6 protein (Figure 17). As shown in Figure 22, the knockdown of SMAD6 in MiaPaCa2 (A) significantly reduced cell replication; conversely, overexpression of SMAD6 in Pa03 (C) significantly increased cell number. SMAD6 knockdown in Panc1 cells also decreased cell replication, albeit to a lesser extent (Figure 22B). While these results suggest that SMAD6 increases cell proliferation, we sought to determine if the degree of apoptosis was at all affected upon SMAD6 modulation. As such, we performed flow cytometry for Annexin V levels in MiaPaCa2 and Panc1 cells in which SMAD6 had been transiently knocked down. SMAD6 knockdown does not,

however, impact the degree of apoptosis in these cell lines, suggesting that its influence on cell number is a result of increased proliferation (Figure 23).

In addition, we sought to determine the effect of SMAD6 on another hallmark of cancer – "activating invasion and metastasis" (184,185). To determine the effect of SMAD6 expression on these properties *in vitro*, we measured the degree to which cells migrate and invade after SMAD6 knockdown or overexpression. In order to measure these properties, we performed migration and invasion assays after transient knockdown or overexpression of SMAD6, as above. Knockdown of SMAD6 significantly impaired the ability of MiaPaCa2 (Figure 24A; $p=0.0003$) and Panc1 (Figure 24B; $p<0.0001$) cells to migrate. Furthermore, knockdown of SMAD6 significantly impaired the ability of MiaPaCa2 (Figure 25A; $p<0.0001$) and Panc1 (Figure 25B; $p<0.0001$) cells to invade through matrigel, while overexpression of SMAD6 promoted the ability of Pa03 cells to invade (Figure 25C; $p=0.01$).

Taken together, these data – that SMAD6 is able to enhance the proliferation, migration, and invasion of pancreatic cancer cells *in vitro* – suggest that SMAD6 is acting as an oncogene in pancreatic cancer.

<u>The clinicopathologic relevancy of SMAD6 in pancreatic cancer.</u>

Our *in vitro* data suggests that SMAD6 promotes aggressive pancreatic cancer behavior. To confirm the relevance of SMAD6 in pancreatic cancer, we performed immunohistochemistry for SMAD6 on primary pancreatic cancers resected at the Johns Hopkins Hospital. Each case was scored to have either "low" or "high" levels of

SMAD6 protein (Figure 18). Interestingly, SMAD6 expression was not correlated with patients' SMAD4 status (Table 17, p=0.158).

In our analyses of our patient cohort, several interesting clinical observations could be gleaned. While SMAD6 status was not correlated to sex, race, age, tumor diameter, differentiation status, node ratio, or N-stage, there were significant associations to T stage and clinical stage: high SMAD6 was associated with stage T2/T3 tumors ($p<0.0001$) with clinical stages 2A and 2B ($p<0.0001$; Table 18). Furthermore, while SMAD6 status was not significantly associated with altered prognosis (p=0.486; Figure 26A), patients whose tumors express high levels of SMAD6 trend toward having reduced survival than those with low levels of SMAD6, especially in patients with stage III disease (p=0.094; Figure 26B).

In addition, immunohistochemistry for SMAD6 was performed on primary pancreatic cancers obtained from pancreatic cancer patients at rapid autopsy. In this cohort, 63.8% of patients' tumors had high SMAD6 expression. SMAD6 levels were, again, not associated with the presence or absence of SMAD4 (Table 19, p=0.74). Although we did not examine the clinical correlates of SMAD6 expression as with our surgical cohort due to the potential compounding variable of treatment, we sought an association between SMAD6 status and metastatic burden. Elevated SMAD6 was linked to widespread metastasis at autopsy, regardless of SMAD4 status (Figure 27, p=0.03). Thus, overexpression of SMAD6 appears to recapitulate the effects seen by SMAD4 loss in pancreatic cancer patients in terms of promoting metastasis (42).

The Effect of SMAD6 on TGF-β and BMP Signaling in Pancreatic Cancer

As previously discussed, the classical function of SMAD6 is to inhibit TGF-β and BMP signaling (74,75). In order to identify the role that SMAD6 plays in modulating these pathways in pancreatic cancer, we sought to determine the effect of SMAD6 overexpression on the activation of these pathways. Given that SMAD6 is a target gene of both TGF-β and BMP (162,163), we used our SMAD6 overexpression system to perform the studies herein in lieu of our knockdown system. We transiently transfected Pa03 and Pa04 cells to overexpress SMAD6 and performed TGF-β and BMP-specific luciferase assays (129,154). To our surprise, there was no significant change in the degree of TGF-β activation upon SMAD6 overexpression (Figure 28A); however, there was a consistent and significant decrease of nearly 40% in the level of BMP response upon SMAD6 overexpression (Figure 28B). In order to confirm the inhibition of BMP signaling by SMAD6 in these cell lines, we performed immunoblotting for the BMP target gene, ID1, in Pa03 and Pa04 cells after SMAD6 overexpression and BMP stimulation. ID1 levels were increased by stimulation with BMP-2, but were diminished upon the addition of SMAD6 (Figure 28C), further corroborating the function of SMAD6 as a BMP inhibitor.

The mechanisms through which SMAD6 has been proposed to inhibit BMP signaling are varied. It has been suggested that SMAD6 is able to bind to type I BMP receptors, specifically ALK-3 and ALK-6 (74,77,186). As a result of these complexes forming, the phosphorylation of downstream R-SMADs is inhibited, potentially via the recruitment of SMURF E3 ubiquitin ligases to degrade both the receptors and the R-SMADs (77). To verify the inhibition of SMAD1/5/8 phosphorylation by SMAD6, we

transiently transfected Pa03 and Pa04 cells with SMAD6 in the presence or absence of BMP-2 ligand and performed immunoblotting for phosphorylated SMAD1/5/8. We observed, however, little noticeable change in the level of phosphorylation of these BMP-associated R-SMADs upon SMAD6 expression (Figure 29A). Others have posited that SMAD6 interferes with BMP signaling by acting as a transcriptional repressor of BMP target genes (76,112,113). We were particularly intrigued by this mechanism due to our observation that SMAD6 is localized in the nuclei of pancreatic cancers in our cohort (Figure 18). To determine if SMAD6, indeed, binds to DNA in pancreatic cancer cells, we transfected MiaPaCa2 and Pa03 cells with a FLAG-tagged SMAD6 construct and performed chromatin immunoprecipitation (ChIP) against the FLAG tag (75). ChIP revealed enrichment of FLAG on the *ID1* promoter in these two cell lines (Figure 29B), confirming that SMAD6 does, indeed, bind to the *ID1* locus in pancreatic cancer cells. Class I histone deacetylases were previously shown to be part of the SMAD6-repressive complex (112); to determine whether histone deacetylation mediates the DNA-binding function of SMAD6, we assayed for *ID1* activation in the presence or absence of the histone-deacetylase inhibitor, Trichostatin A (187). As determined by luciferase assay, inhibition of histone deacetylases does not affect the degree to which *ID1* is transcribed (Figure 29D). Therefore, the mechanism of SMAD6 in the nucleus appears to be independent of histone deacetylases in pancreatic cancer, although SMAD6 does appear to be interfering with BMP-induced *ID1* expression through acting as a transcriptional repressor.

Therefore, we have shown the SMAD6 increases the aggressiveness of pancreatic cancer and inhibits BMP signaling. To ascertain that these effects are linked, we assessed

the effects of BMP modulation on MiaPaCa2 and Pa03 cells through activation of BMP (to mirror SMAD6 knockdown) or inhibition of BMP (to mimic SMAD6 overexpression). Knockdown of SMAD6 in MiaPaCa2 yielded decreased proliferation (Figure 22A) and invasion (Figure 25A); we predicted stimulation of these cells with BMP to yield similar changes. However, BMP promoted proliferation of MiaPaCa2 cells (Figure 30A) and did not significantly affect invasion (Figure 30C). Overexpression of SMAD6 in Pa03 cells increased both proliferation (Figure 22C) and invasion (Figure 25C). Yet, treatment of Pa03 with a BMP-2/4 neutralizing antibody failed to significantly impact either behavior (Figure 30B, D). These data suggest that BMP inhibition is not the mechanism through which SMAD6 impacts pancreatic cancer. To further confirm this observation, we overexpressed SMAD6 in Pa01 cells, which harbor a homozygous deletion of SMAD4 (21) and have no measurable BMP signaling (Figure 14B). Since there is no BMP signaling to inhibit, we would expect overexpression of SMAD6 to have no effect of these cells. In spite of this hypothesis, SMAD6 overexpression significantly increased the migration of Pa01 (Figure 30F; $p=0.0002$), although proliferation was not affected (Figure 30E). Thus, SMAD6 impacts cell behavior independently of its ability to inhibit BMP.

In light of our observations that SMAD6 performs outside of TGF-β and BMP inhibition in pancreatic cancer, we sought to determine its true function in the disease. In addition to inhibiting TGF-β and BMP signaling, SMAD6 has been proposed to inhibit Wnt and NFκB signaling (117,118,188). However, we have shown that SMAD6 acts to promote pancreatic cancer aggressiveness, whereas these two pathways also are considered to be oncogenic in this disease (189-192). Therefore, it is unlikely that

SMAD6 is acting through inhibition of either of these networks. As previously discussed, that SMAD6 is able to bind to DNA and repress transcription (76,112,113), and we have confirmed that SMAD6 binds to the promoter of the BMP target gene, *ID1* (Figure 29B).

In order to gain a better understanding of the nuclear function of SMAD6, we performed subcellular fractionation of MiaPaCa2 and Panc1, the two cell lines that overexpress SMAD6, in the presence or absence of BMP-2 ligand. We then assessed the levels of SMAD6 in the cytoplasm and nucleus of these lysates. As shown in Figure 31A, SMAD6 is constitutively present in the nuclei of these cell lines, and remains largely unchanged upon BMP-2 stimulation. To confirm the perpetual presence of SMAD6 in the nucleus of pancreatic cancer cell lines, we repeated the subcellular fractionation experiments using cell lines that had been transiently transfected to overexpress SMAD6. In Pa03 and Pa04 cells, SMAD6 was, again, expressed in both the cytoplasm and nucleus regardless of BMP stimulation (Figure 31B). These results are in agreement with the observations of Bai *et al*. and Lin *et al.* (76,113) and further the notion that the nuclear function of SMAD6 in pancreatic cancer does not require BMP. Thus, the nuclear function of SMAD6 is likely, to some degree, separate from its BMP inhibiting capacity.

Discussion

SMAD6 has classically been described as an inhibitor of TGF-β and BMP signaling (74,75). As such, one would expect its overexpression in pancreatic cancer to mirror the effects of SMAD4 loss, as the latter is a common mediator to both TGF-β and BMP pathways (36). Indeed, in this chapter, we have demonstrated that SMAD6 promotes proliferation, to some degree, (Figure 22), migration (Figure 24), and invasion (Figure 25) of pancreatic cancer cells *in vitro* and increases the level of metastasis in pancreatic cancer patients (Figure 27). Previous research has indicated that SMAD6 promotes aggressive behavior in lung cancer cells (111). Therefore, it appears that SMAD6 overexpression at least partially phenocopies SMAD4 loss and is an oncogenic event in pancreatic cancer.

Thus, as discussed in Chapter 4, SMAD6 is likely targeted for degradation under normal conditions; this ubiquitination is, then, lost to cause increased protein expression in a subset of pancreatic cancer (Figure 20). Given the degree to which ubiquitination is disrupted in human cancer, there has been interest in targeting the Ubiquitin-proteasome pathway as part of cancer treatment. Bortezomib, which inhibits the 20S proteasome, is currently FDA-approved for the treatment of several cancer types, and there are new drugs in this class in development (reviewed in (193)). However, one must wonder about the efficacy of such agents. Given the effects of SMAD6 overexpression in pancreatic cancer caused by a loss of this protein's degradation, one would presume that inhibition of the proteasome, in general, would result in the aberrant activation of SMAD6 and other known oncogenes. Therefore, it would seem that more specific drugs, such as

nutlins, which disrupt MDM2-mediated TP53 degradation (194), would be less risky forms of cancer treatment.

Of the four principal genes known to be mutated in pancreatic cancer – *KRAS, CDKN2A, TP53*, and *SMAD4* – only *KRAS* contains a gain-of-function mutation. As such, oncogenic KRAS is the only known pancreatic cancer driver that is, in theory, directly targetable. However, attempts to target KRAS have been unsuccessful, to date. Recently, screens for drugs that are synthetically lethal in the presence of *KRAS* mutations were unsuccessful in pancreatic cancer cells (141). Accordingly, there has been significant interest in the determination of alternative targets in pancreatic cancer. SMAD6 could fit this profile. It is present in 70.6% of pancreatic cancers studied herein, and is able to enhance proliferation (Figure 22), migration (Figure 24), and invasion (Figure 25) *in vitro*, while it is associated with tumor stage in in pancreatic cancers at surgery (Table 18) and correlates with increased metastasis at autopsy (Figure 27). While SMAD6 status was not indicative of a difference in survival in patients overall (Figure 26A), in late-stage cancers (Stage III), there is a trend toward a disadvantage in survival for SMAD6-high patients (Figure 26B). Given the low percentage of patients whose tumors do not overexpress SMAD6, it is possible that the expansion of our cohort will yield more conclusive evidence about the role of SMAD6 in pancreatic cancer prognosis.

Despite our observations in this chapter that SMAD6 contributes to the development of pancreatic cancer, the mechanism through which SMAD6 acts in pancreatic cancer is, as of yet, unknown. In a previous study in non-small cell lung cancer, Jeon *et al.* identified many pathways and processes that were altered upon SMAD6 knockdown, including cell cycle, cell proliferation, and cellular movement,

which support our experimental observations (111). In order to further study the mechsnism of SMAD6 action in pancreatic cancer, we first assessed its contribution to inhibition of TGF-β superfamily signaling. While prior studies have indicated that SMAD6 inhibits TGF-β signaling in pancreatic cancer (110), we sought to verify whether SMAD6 affects TGF-β, BMP, or both pathways in our pancreatic cancer model. Through the use of luciferase reporters specific for both networks, we conclude that BMP is the target pathway of SMAD6 in pancreatic cancer cells, rather than TGF-β (Figure 28); we were able to validate these data through immunoblotting for the BMP target gene, ID1, in the presence or absence of SMAD6 and BMP-2 (Figure 28C). While SMAD6 did not affect the degree to which BMP-associated R-SMADs were phosphorylated (Figure 29A), we were able to detect SMAD6 on the promoter of *ID1* via ChIP (Figure 29B). Previous work has shown that SMAD6 is able to bind to DNA through its MH1 domain and repress BMP-mediated transcription via complexing with histone deacetylases (HDACs) and additional transcriptional repressors, such as HOXC8 and CTBP (76,112,113). However, we were not able to associate the SMAD6-mediated repression of ID1 transcription with HDAC function (Figure 29D). Still, it appears that SMAD6 is modulating BMP responses through repression of target gene transcription in pancreatic cancer.

In order to further study the association between BMP signaling and SMAD6 in pancreatic cancer, we sought to mimic SMAD6 overexpression through inhibition of BMP signaling. In Pa03 cells, overexpression of SMAD6 led to an increase in proliferation and invasion *in vitro* (Figure 22D, Figure 25C). However, inhibition of BMP signaling in these cells did not yield a significant change in either behavior (Figure

30B,D). Similarly, in MiaPaCa2, in which SMAD6 knockdown reduced proliferation and invasion (Figure 22A, Figure 25A), activation of BMP signaling did not affect the invasive potential (Figure 30C), while it increased the proliferative rate (Figure 30A). Limited prior studies have examined the effect of BMP on pancreatic cancer cell behavior. However, activation of BMP has previously been associated with more aggressive behavior of Panc1 cells (105-107). We have shown SMAD6 knockdown to result in the opposite effects in this cell line (Figure 22B, Figure 24B, Figure 25B). To further delineate the relationship between SMAD6 and BMP signaling in pancreatic cancer, we determined the effects of SMAD6 expression in the SMAD4-null cell line, Pa01 (21). While SMAD6 did not affect the proliferation of Pa01 cells (Figure 30E), it significantly increased these cells' migratory capacity (Figure 30F; $p=0.0002$). Finally, we noted that the expression of SMAD6 in patients was not predicated on the presence of SMAD4 (Table 17, Table 19). These data caused us to question whether BMP inhibition is the mechanism through which SMAD6 functions in pancreatic cancer.

While the BMP-specific functions of SMAD6 in the nucleus are not likely to be affecting pancreatic cancer cell behavior, we remain intrigued by the nuclear function of this protein. We and others have shown that SMAD6 is constitutively located in the nucleus, regardless of BMP stimulation (76,113) (Figure 31). While it has been shown that SMAD6 represses BMP-mediated transcription, the possibility remains that there are other target genes that SMAD6 regulates in a BMP-independent manner. Further work will attempt to clarify the BMP-independent nuclear mechanism of SMAD6 in pancreatic cancer.

In conclusion, we have determined that SMAD6 overexpression is associated with aggressive properties of pancreatic cancer – proliferation, migration, and invasion – *in vitro*, while we have associated SMAD6 protein levels with elevated metastasis in pancreatic cancer patients. However, the mechanism through which SMAD6 appears to be acting is not through its canonical role of BMP inhibition. Rather, we sought to determine if the presence of SMAD6 in the nucleus of pancreatic cancer cells significantly impacted pancreatic cancer biology. Given that SMAD6 has been shown to have the capacity to repress transcription, it is worth noting that the levels of ID1 are reduced upon SMAD6 overexpression and SMAD6 binds to the promoter region of this gene. We have determined that SMAD6 is constitutively present in the nucleus of pancreatic cancer cells. Further study will clarify the nuclear role of SMAD6 in relationship to its oncogenic properties in pancreatic cancer.

Table 14. Antibodies used for immunoblotting.

Antibody	Company	Dilution
SMAD6	Imgenex (San Diego, CA)	1:500
ID1	Santa Cruz Biotechnology (Santa Cruz, CA)	1:500
p-SMAD1/5/8	Cell Signaling (Danvers, MA)	1:1000
t-SMAD1/5/8	Santa Cruz Biotechnology (Santa Cruz, CA)	1:1000
LAMIN A/C	Cell Signaling (Danvers, MA)	1:1000
GAPDH	Cell Signaling (Danvers, MA)	1:5000
HRP-linked anti-rabbit IgG	GE Healthcare (Piscataway, NJ)	1:5000

Table 15. Antibodies used for immunohistochemistry.

Antibody	Company	Dilution
SMAD6	Invitrogen (Carlsbad, CA)	1:500
SMAD4	Santa Cruz Biotechnology (Santa Cruz, CA)	1:100

Table 16. Primer sequences used for Chromatin Immunoprecipitation

Primer Name	Primer Sequence
ID1 ChIP Forward	5- ACAGTCCGTCCGGGTTTTAT -3
ID1 ChIP Reverse	5- CCCATTTTTGGCTGCTTTT -3

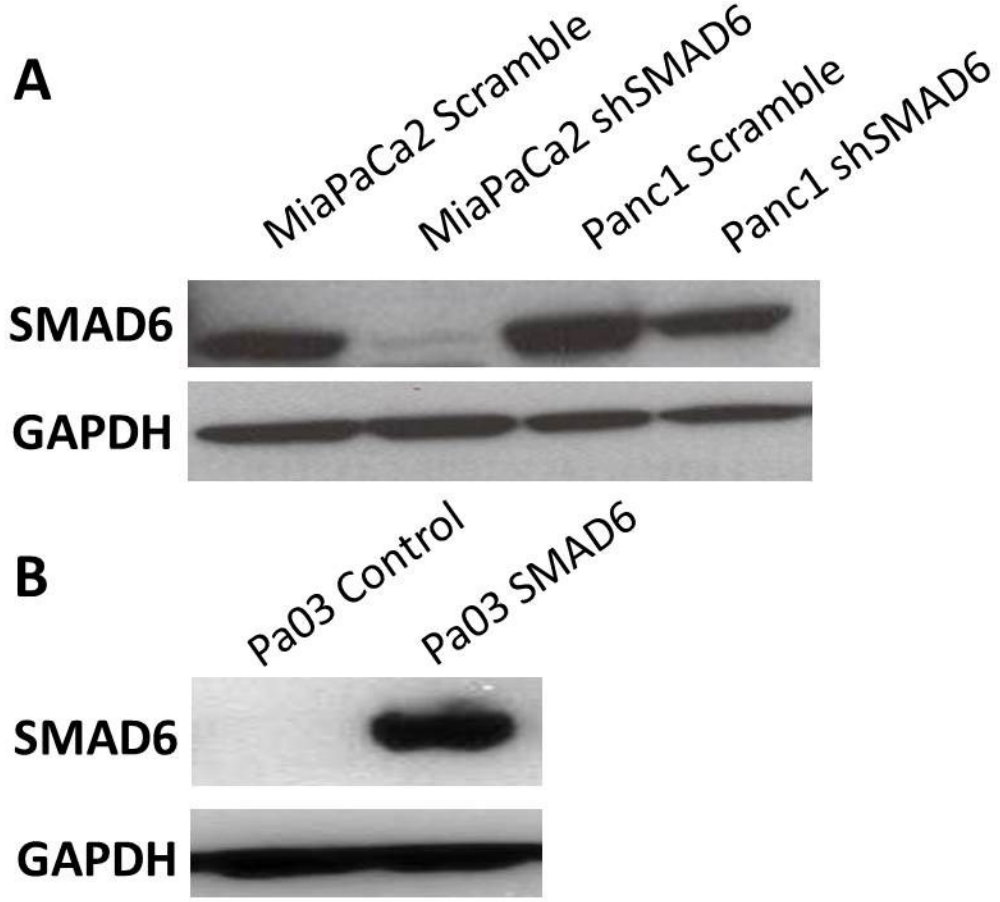

Figure 21. SMAD6 modulation in pancreatic cancer cell lines.
Transient modulation of SMAD6 in MiaPaCa2, Panc1, and Pa03 cells were verified 72 hours after transfection by immunoblotting for SMAD6. SMAD6 was successfully A) knocked down and B) overexpressed in these lines. Expression is normalized to GAPDH.

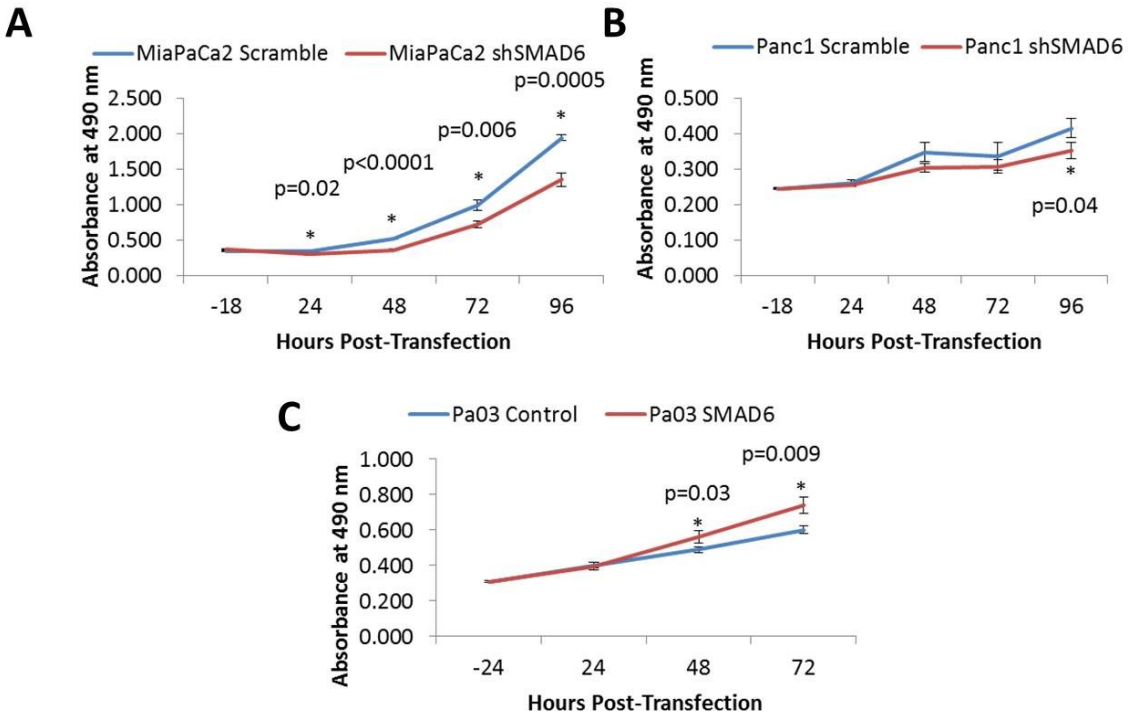

Figure 22. Effect of SMAD6 on pancreatic cancer cell proliferation.
Differences in cell viability were determined by MTT assay after SMAD6 knockdown. Knockdown of SMAD6 reduces cell viability in A) MiaPaCa2 and, to a lesser extent, B) Panc1 cells. C) Differences in cell viability were determined by MTT assay after SMAD6 overexpression. Overexpression of SMAD6 increases cell viability in Pa03 cells. Significance was calculated by Student's t-test. All error bars represent standard deviation.

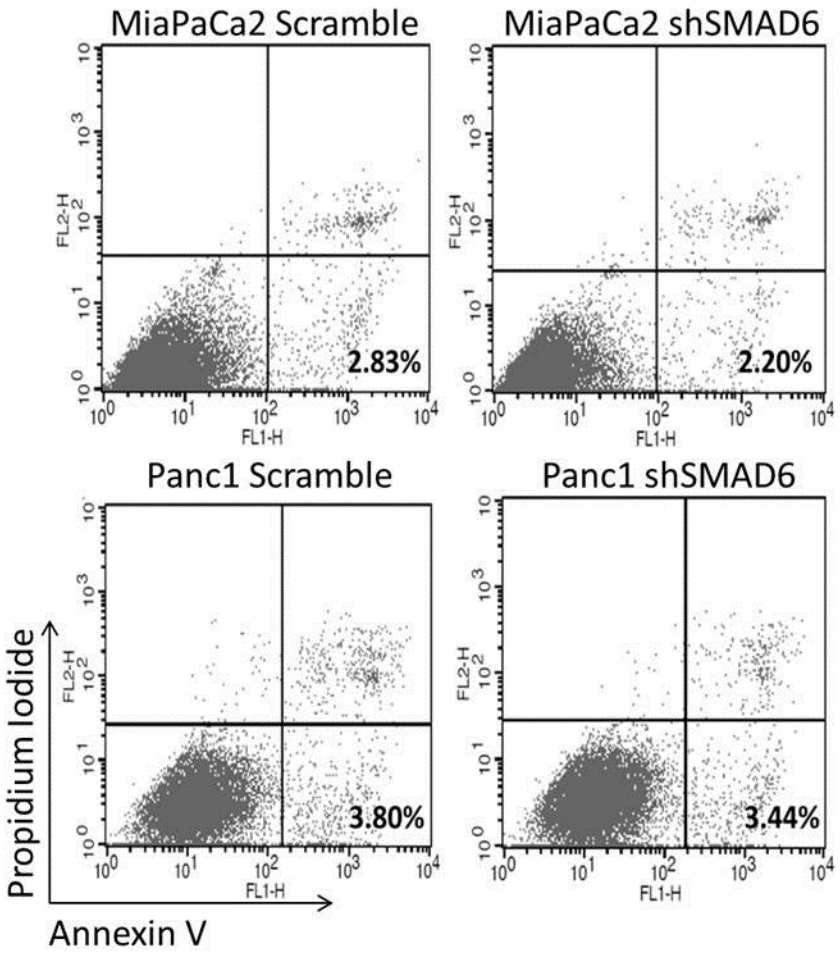

Figure 23. Effect of SMAD6 on apoptosis.
The levels of apoptosis were determined in MiaPaCa2 and Panc1 cells 72 hours after transfection of shSMAD6 by flow cytometry for Annexin V. There is little change in the degree of apoptosis upon SMAD6 knockdown.

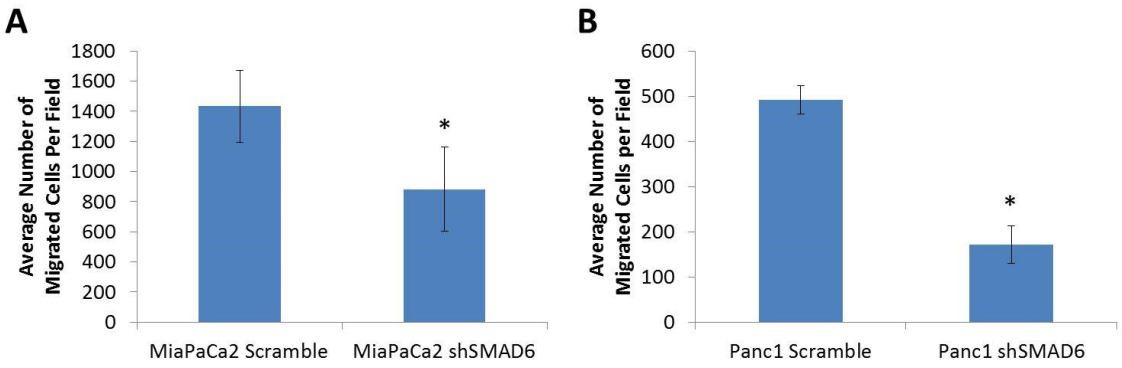

Figure 24. Effect of SMAD6 on pancreatic cancer cell migration.
Differences in cell migration capacity were determined after SMAD6 knockdown. For both A) MiaPaCa2 and B) Panc1, cells displayed reduced migration after SMAD6 knockdown ($p=0.0003$ and <0.0001, respectively; Student's t-test). All error bars represent standard deviation.

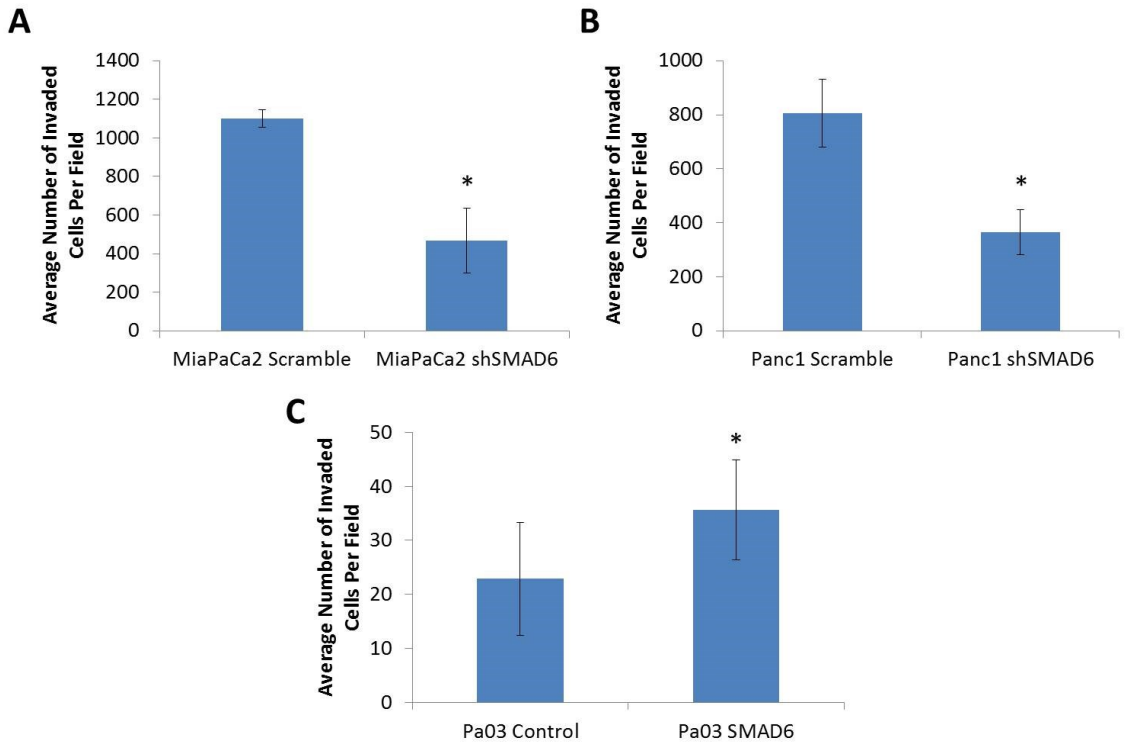

Figure 25. Effect of SMAD6 on pancreatic cancer cell invasion.
Differences in cell invasion capacity were determined after SMAD6 knockdown. For both A) MiaPaCa2 and B) Panc1, cells displayed reduced invasion after SMAD6 knockdown ($p < 0.0001$ for both; Student's t-test). C) Differences in cell invasion capacity were determined after SMAD6 overexpression. Cells overexpressing SMAD6 displayed a significantly greater invasion than control cells ($p=0.01$; Student's t-test). All error bars represent standard deviation.

Table 17. Relationship between SMAD4 status and SMAD6 status in primary pancreatic cancers.

	Low SMAD6 N=(27)	**High SMAD6 (N=52)**	**p**
SMAD4 Status			
Intact	14	29	0.158
Lost	13	23	

* Significance was calculated using Chi-square analysis.

Table 18. Clinicopathologic features of SMAD6 expression surgically resected pancreatic cancers.

	Low SMAD6 (N=40)	High SMAD6 (N=104)	p
Age at diagnosis (years)	63.60 ± 12.683	67.76 ± 10.843	0.105
Sex			
Male	22	59	0.499
Female	18	45	
Race			
White	35	92	0.336
Black	4	5	
Other	1	7	
Tumor Diameter (cm)	3.358 ± 1.4381	3.505 ± 1.4538	0.339
Differentiation			
Well	1	2	0.535
Moderate	22	47	
Poor	17	55	
Clinical Stage			
1A	1	0	<0.001
1B	0	1	
2A	3	14	
2B	16	74	
3	20	15	
T Stage			<0.001
T1	5	10	
T2/T3	15	79	
T4	20	15	
N Stage			
N0	5	16	0.441
N1	35	88	
Node Ratio			
Less than 0.3	22	71	0.098
Greater than 0.3	18	33	

* Significance was calculated using student's t-test (age and tumor diameter), Fisher exact analysis (race, differentiation, clinical stage, T stage, and N stage), and Chi-square analysis (sex and node ratio).

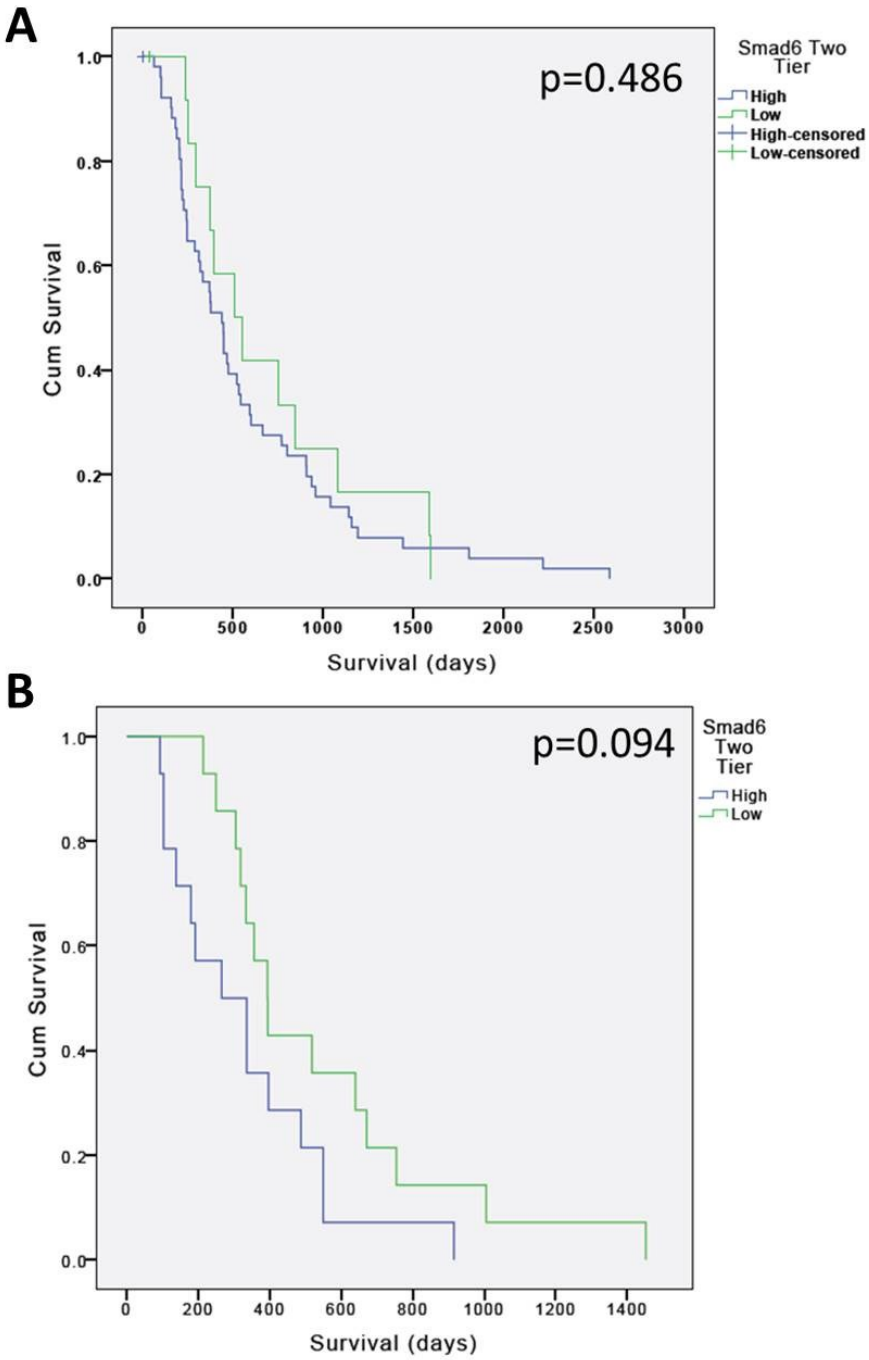

Figure 26. The impact of SMAD6 on pancreatic cancer prognosis.
A) Kaplan-Meier analysis of our cohort of surgically resected pancreatic cancers indicates no association between SMAD6 status and survival. B) Kaplan-Meier analysis of the Stage III patients alone suggests that SMAD6 may confer a survival disadvantage on patients in late-stage pancreatic cancer.

Table 19. Relationship between SMAD4 status and SMAD4 status in primary pancreatic cancers at rapid autopsy.

	Low SMAD6 N=(13)	**High SMAD6 (N=23)**	**p**
SMAD4 Status			
Intact	8	13	0.74
Lost	5	10	

* Significance was calculated using Fisher exact analysis.

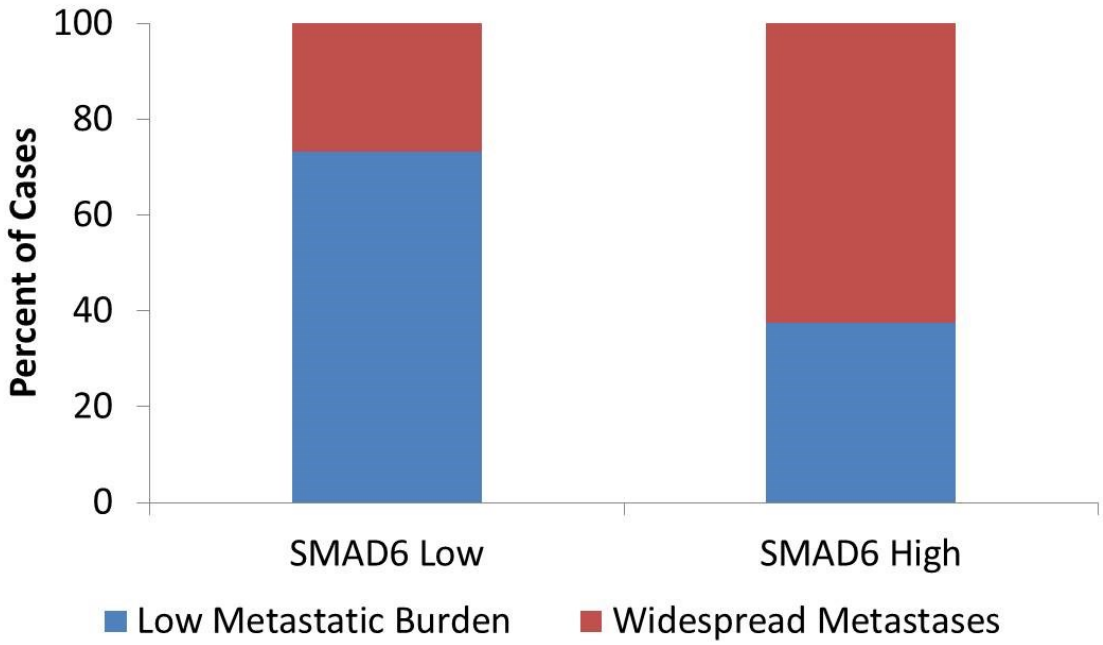

Figure 27. The impact of SMAD6 on pancreatic cancer metastasis.
Immunohistochemistry for SMAD6 was performed on pancreatic cancers from autopsy patients. The degree of metastasis at autopsy (Low – fewer than 100 metastases; Widespread – greater than 100 metastases) was correlated to the level of SMAD6 expression. High SMAD6 expression was associated with widely metastatic disease ($p=0.03$; Chi-square analysis).

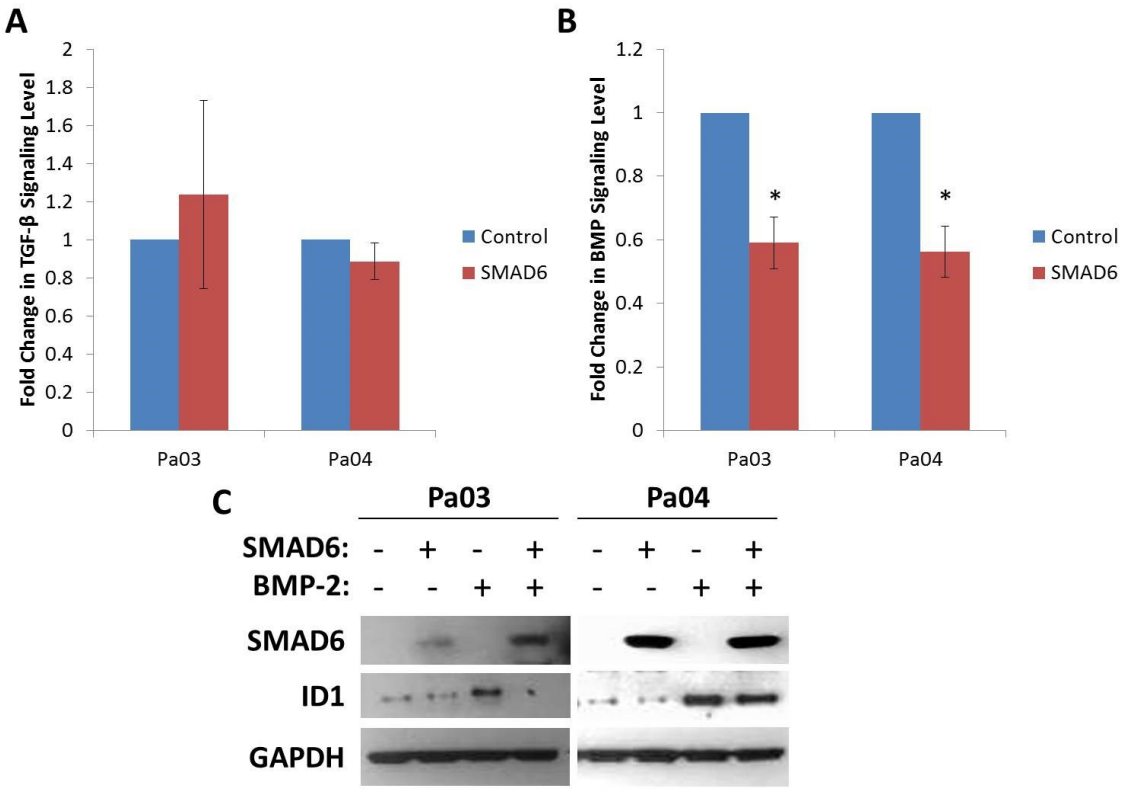

Figure 28. Effect of SMAD6 on TGF-β and BMP activity.
Pa03 and Pa04 cells were transfected for SMAD6 overexpression, along with luciferase reporters specific for A) TGF-β and B) BMP response. Data are presented such that the wild-type to mutant promoter ratio is set to 1. Pathway activity is presented as the fold increase in luciferase levels after addition of ligand (e.g. SBE + TGF-β/mSBE + TGF-β). Error bars represent standard error of the mean. C) Pa03 and Pa04 cells were transfected for SMAD6 overexpression and stimulated with BMP-2 ligand. ID1 levels are increased upon BMP-2 stimulation, but decrease in the presence of SMAD6.

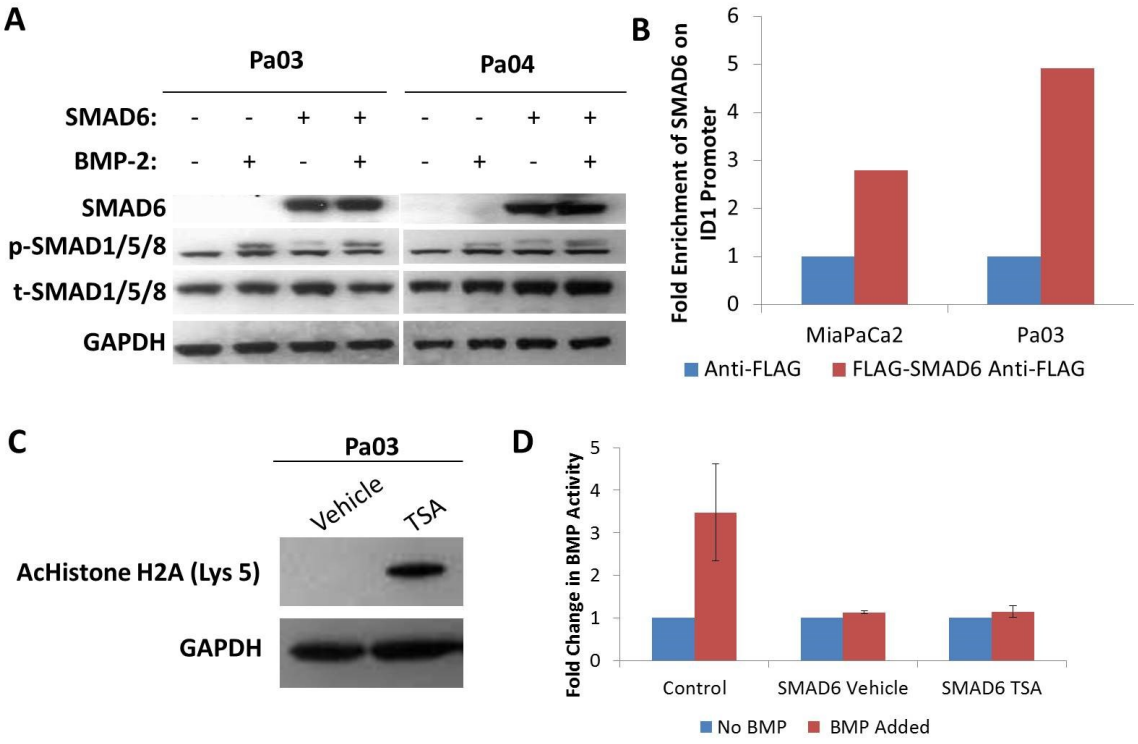

Figure 29. SMAD6 binds to the *ID1* promoter.
A) Pa03 and Pa04 cells were transfected for SMAD6 overexpression and stimulated with BMP-2 ligand. The level of p-SMAD1/5/8 was measured by immunoblotting; phosphorylation of SMAD1/5/8 remains unchanged upon expression of SMAD6. B) MiaPaCa2 and Pa03 cells were transfected with CS2-FLAG-SMAD6 or left untransfected. Chromatin immunoprecipitation was performed against the FLAG epitope in both conditions. Enrichment of SMAD6 on the *ID1* promoter was determined using quantitative real-time PCR and compared to a pull-down of the FLAG-epitope in the untransfected cells. C) Treatment of Pa03 cells with 10 ng/mL trichostatin A (TSA) results in a buildup of Acetylated Histone H2A. D) SMAD6 was transiently overexpressed in Pa03 cells. These cells were treated with either DMSO (Vehicle) or TSA and were simultaneously stimulated with BMP-2 ligand. Treatment with TSA did not significantly change the level of *ID1* transcription, measured by luciferase reporter. Error bars represent standard error of the mean.

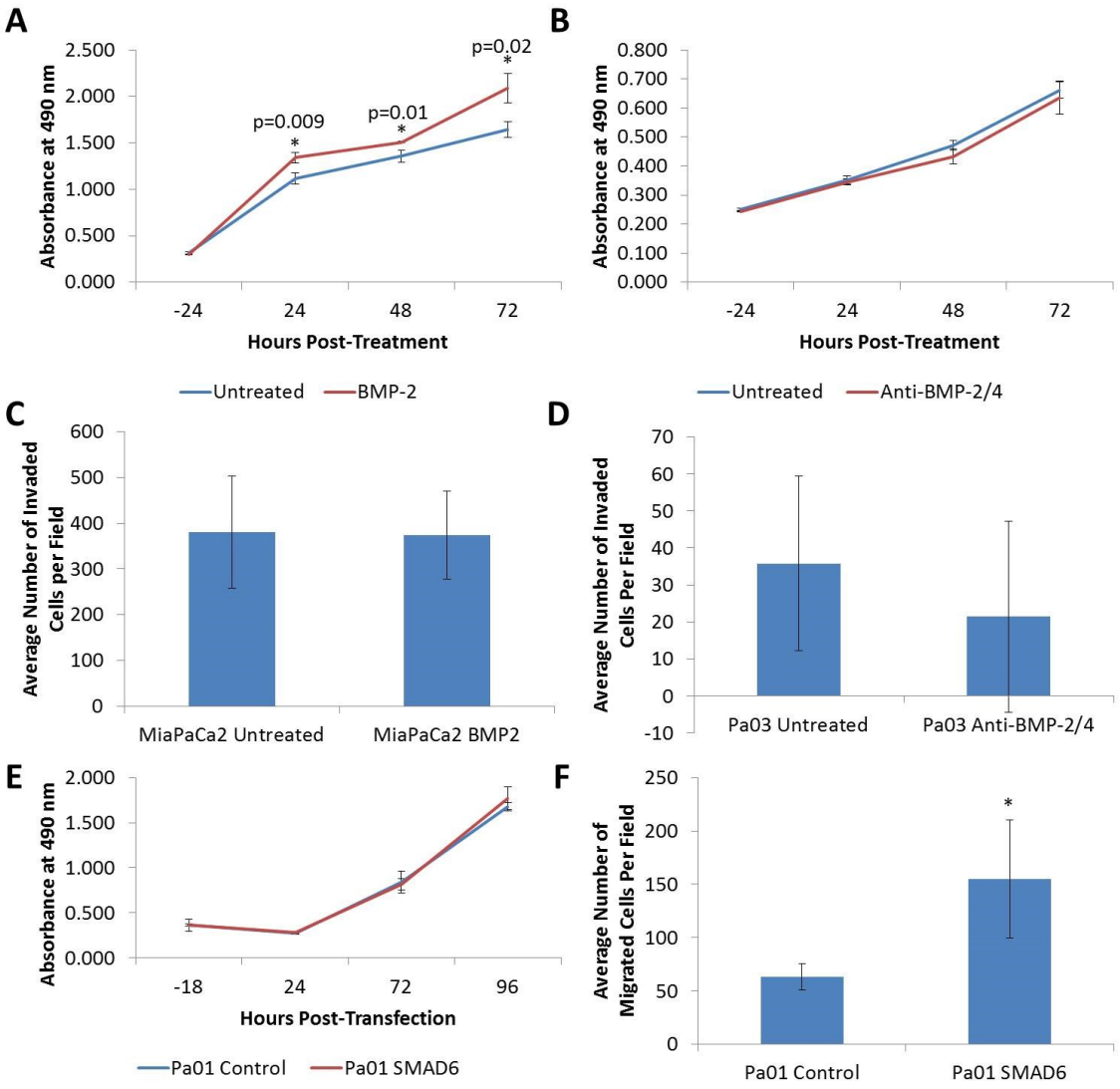

Figure 30. Effects of BMP on pancreatic cancer cell behavior.
A) Differences in cell viability before and after BMP stimulation were determined by MTT assay in MiaPaCa2 cells. BMP treatment did not phenocopy SMAD6 knockdown: BMP-2 treatment increased cell viability. B) Differences in cell viability with and without BMP blockade was determined by MTT assay in Pa03 cells. BMP inhibition did not phenocopy SMAD6 overexpression: BMP-2/4 inhibition had no effect on cell viability. C) Differences is cell invasiveness before and after BMP stimulation were determined in MiaPaCa2 cells. BMP treatment did not phenocopy SMAD6 knockdown: BMP-2 treatment had no effect on invasion. D) Differences in cell invasiveness with and without BMP blockade was in Pa03 cells. BMP inhibition did not phenocopy SMAD6 overexpression: BMP-2/4 inhibition had no effect on invasion. E) Differences in cell viability were determined by MTT assay after SMAD6 overexpression. Overexpression of Smad6 increases cell viability in Pa03 cells. F) Differences in cell migration capacity were determined after SMAD6 overexpression. For Pa01, cells displayed increased migration after SMAD6 knockdown ($p=0.0002$). All significance was calculated by Student's t-test. All error bars represent standard deviation.

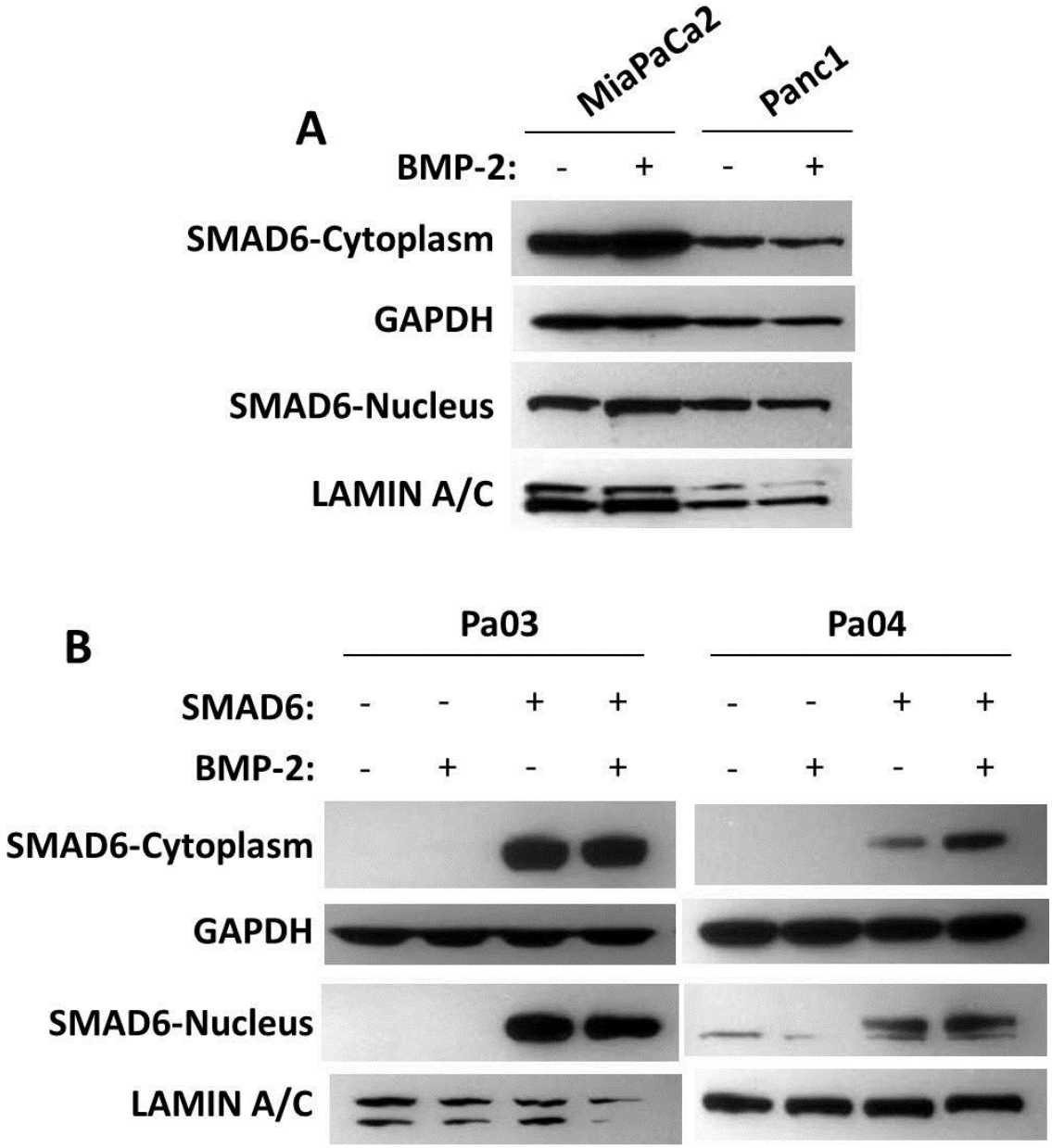

Figure 31. Baseline levels of SMAD6 in the cytoplasm and nucleus of pancreatic cancer cells.

The cellular localization of SMAD6 in MiaPaCa2 and Panc1 cells (A) and Pa03 and Pa04 Control and SMAD6 cells (B) was determined by subcellular fractionation followed by immunoblotting for SMAD6 with and without BMP stimulation. Cells were serum-starved for 24 hours prior to treatment with BMP-2. Both native and overexpressed SMAD6 was constitutively present in the nucleus.

CHAPTER 6. CONCLUSIONS AND FUTURE DIRECTIONS

As a result of our observation that SMAD4 loss was associated with an increased metastatic burden in pancreatic cancer (42), our studies were aimed at deciphering the mechanism through which loss of TGF-β and BMP signaling led to this clinical phenomenon. As such, we focused, specifically, on the effects of SMAD4 loss as well as, broadly, the roles of TGF-β and BMP signaling in pancreatic cancer. Through the latter, we identified SMAD6, previously characterized as an inhibitor of these pathways (74,75), as a protein of interest in pancreatic cancer.

SMAD4

In order to better understand the role of SMAD4 in pancreatic cancer metastasis, we generated isogenic cell lines (both parental "pooled" lines and those derived from single cells) for SMAD4 expression (Figure 2, Figure 4). Use of these isogenic cell lines yielded interesting observations regarding the effect of SMAD4 on cell behavior *in vitro*. Cells re-expressing SMAD4 exhibited a reduced proliferation rate (Figure 5) as well as degrees of migration (Figure 6A) and invasion (Figure 6B). These properties support previous xenograft studies indicating that SMAD4 loss increases tumor growth and angiogenesis (89-91), as well as the clinical observations that SMAD4 loss portends a shortened survival (93,94) and increased metastasis (42). Still, these conclusions about such an important component of TGF-β and BMP signaling disagree with the findings that stimulation with TGF-β and BMP promotes an epithelial-mesenchymal transition *in vitro*, a property considered to be reminiscent of a metastatic phenotype (95-97,105-107). As such, we attempted to rationalize these seemingly conflicting data with the hypothesis

that SMAD4 loss reroutes the TGF-β/BMP signal through an alternative, oncogenic pathway. Sure enough, screening our pooled isogenic cell lines for differential pathway activation indicated that the ERK and Pi3K pathways were preferentially activated by TGF-β in the absence of SMAD4 (Figure 3). However, we were unable to confirm these observations in a cohort of surgically resected pancreatic cancers with known SMAD4 status (Table 5, Table 6). Additional study will, hopefully, shed light on whether there is an alternative pathway that is activated by TGF-β ligands in the absence of SMAD4 in pancreatic cancers. There are a number of additional pathways that have been reported to act as SMAD-independent TGF-β responses (123-126,128). Furthermore, it is possible that our patient cohort was too small to capture a true relationship, if any, between SMAD4 status and activation of ERK and Pi3K in pancreatic cancers.

Given that it is lost in approximately half of pancreatic cancers (33), SMAD4 would be an excellent marker to guide treatment of this disease. In collaboration with Drs. Yunfeng Cui and James Eshleman, we tested this hypothesis *in vitro* and we published these results (131). An association was made between cell lines of known SMAD4 status and the efficacy of irinotecan (Figure 10, Table 7, Table 8) and cisplatin (Figure 11A, Table 7, Table 8). In order to further confirm these observations, SMAD4-isogenic cell lines, described above, were used to study the ability of these agents to kill pancreatic cancer cells in the preferential presence or absence of SMAD4. As shown in Figure 12 and Table 9, cells with loss of SMAD4 were more sensitive to cisplatin and irinotecan and more resistant to gemcitabine. These phenomena, particularly those of cisplatin and gemcitabine, were correlated with the degree to which the TGF-β pathway was active (Figure 13, Table 10). While the fold changes in resistance that we observed

are statistically significant, it must be noted that they are small. Further study will be required to parse out the true utility of SMAD4 status as a guide for chemotherapy treatment. Mouse experiments are planned to validate these results *in vivo*.

SMAD6

In an attempt to better understand the nature of TGF-β and BMP signaling in pancreatic cancer, we performed luciferase assays specific for these pathways on a panel of pancreatic cancer cell lines that had been identified as having no mutations in pathway components. As shown in Figure 14, there was a high degree of variability in the activities of these pathways in these cell lines, with many cell lines displaying little to no signaling activity. We, therefore, sought to determine a rationale for the non-genetic inhibition of TGF-β and BMP. Despite reports to the contrary in other cell types (57,157,158), we found neither oncogenic KRAS nor downstream MEK signaling to significantly impinge on the activation of these pathways in our pancreatic cell lines (Figure 15, Figure 16). Rather, through a screen of known TGF-β and BMP antagonists, we identified SMAD6 as a promising candidate for further study, based on its extreme differential expression in our cell lines and, subsequently, in pancreatic cancer tissue (Figure 17, Figure 18).

Interestingly, several sequencing studies have been completed in pancreatic cancer, comprising 135 patients, and not a single SMAD6 mutation was identified (21,22,161). As such, we concluded that the regulation of SMAD6 must be epigenetic. SAGE data indicates that the expression of *SMAD6* transcript is variable among

pancreatic cancers (21). However, in our cell line panel, the levels of *SMAD6* transcript did not correspond to the level of SMAD6 protein (Figure 19), indicating that transcriptional control is not the means for the differential regulation of SMAD6. Rather, we found that treatment of SMAD6-negative cell lines with MG132, a proteasome inhibitor (164), restored the expression of SMAD6 (Figure 20). Therefore, we conclude that SMAD6 is regulated through aberrant proteolytic degradation. The underlying cause for this phenomenon, however, remains unclear. Further study will illuminate the factor or factors responsible for the loss of SMAD6 degradation in pancreatic cancer; recently, a screen for SMAD6 binding partners was completed, and several proteins that function in modulation of ubiquitination were determined to bind SMAD6 (171). It is conceivable that one or many of these factors is responsible for the aberrant expression of SMAD6 in pancreatic cancer, with the possibility that there exist patient-specific regulatory errors in this process.

Given that, in theory, SMAD4 loss and SMAD6 elevation would both result in loss of TGF-β and BMP signaling, we hypothesized that the increased SMAD6 expression would phenocopy SMAD4 loss. Indeed, we have associated SMAD6 expression with an increase in migration (Figure 24) and invasion (Figure 25); proliferation also appears to be impacted by SMAD6, although to a lesser extent in Panc1 than in MiaPaCa2 and Pa03 (Figure 22). Furthermore, we have shown a link between elevated SMAD6 expression and metastatic burden in pancreatic cancer patients at autopsy, similar to SMAD4 loss (42) (Figure 27). We find it noteworthy that the clearest effects of SMAD6 on pancreatic cancer cell behavior (migration and invasion) most closely mirror the most significant clinical correlation of SMAD6 expression: increased

metastasis (Figure 27). However, the association that we have made between SMAD6 and metastasis is correlative, at this point. We aim to perform *in vivo* assays using SMAD6-modulated cell lines (overexpressed or knocked down) to determine if SMAD6 expression can promote metastasis. In addition, given the associations that we have made between SMAD6 protein levels and cell behavior, it is our hope that SMAD6 can be studied in the context of chemosensitivity. Given the high degree to which it is expressed in pancreatic cancer, there would be a great clinical boon to determining if SMAD6 can be a marker for guiding therapy.

Given the seemingly parallel downstream effects of SMAD4 loss and SMAD6 elevation, we hypothesized that it was through inhibition of TGF-β superfamily signaling that SMAD6 exerts its effects in pancreatic cancer. In order to validate that SMAD6, indeed, functions through the inhibition of TGF-β and/or BMP signaling in pancreatic cancer, we performed luciferase assays specific for these pathways on cell lines in which SMAD6 was transiently overexpressed. It appears that SMAD6 does not significantly impact TGF-β signaling (Figure 28A) but, instead, inhibits the BMP response (Figure 28B). However, when we analyzed the inhibition of SMAD1/5/8 phosphorylation, SMAD6 did not seem to have an effect (Figure 29). Rather, SMAD6 binds to the promoter region of *ID1* (Figure 29B) and has previously been shown to inhibit BMP-mediated transcription (76,112,113).

Subsequently, we aimed to verify that BMP inhibition was the mechanism through which SMAD6 conferred behavioral changes upon pancreatic cancer cells. Unexpectedly, stimulation or inhibition of BMP activity in MiaPaCa2 and Pa03 cells did not yield similar changes in proliferation (Figure 30A-B) and invasion (Figure 30C-D) as

did SMAD6 modulation. Furthermore, SMAD6 overexpression yielded functional changes in the absence of SMAD4 – overexpression of SMAD6 increased the migratory capacity of Pa01, a cell line with a homozygous deletion of SMAD4 (21) (Figure 30F). Thus, we conclude that SMAD6 acts independently of BMP inhibition in the biology of pancreatic cancer.

While the canonical function of SMAD6 has been the inhibition of BMP signaling, there have been other functions ascribed to this protein. Notably, as aforementioned, SMAD6 has been shown to have transcriptional repressive capability (76,112,113). We observed a striking trend in our immunohistochemical analyses of SMAD6: cases in which SMAD6 was overexpressed displayed strong nuclear positivity (Figure 18). In agreement with studies by Bai *et al.* and Lin *et al.*, we were able to demonstrate that SMAD6 is constitutively present in the nuclei of pancreatic cancer cells, regardless of stimulation with BMP ligand (Figure 31). That SMAD6 is constitutively present in the nucleus suggests that it has nuclear functions separate from inhibiting BMP responses. We hypothesize, therefore, that the BMP-independent actions of SMAD6 in the nuclei of pancreatic cells is the mechanism that contributes to altered pancreatic cancer cell behavior.

In an attempt to further understand the nuclear role of SMAD6, we performed ChIP-seq analysis on MiaPaCa2 and Pa03 cells expressing FLAG-SMAD6 (Figure 29B) to identify novel, BMP-independent binding regions in the genome. However, the data were, largely, inconclusive. Still, there were several putative peaks identified in both MiaPaCa2 and Pa03 cells (data not shown). Of the peaks identified, one target, *ErbB4*, is of particular note. The neuregulin pathway, of which ERBB4 is a member (195), was

identified in a previous microarray study as being one of the major pathways impacted by SMAD6 modulation (111). Given the putative function of SMAD6 as a transcriptional repressor, it is of note that ERBB4 expression is reduced in pancreatic cancer compared to normal pancreatic tissue, and its expression was not detected in any metastatic pancreatic cancers analyzed (196,197). These results align with our observation that increased SMAD6 expression correlates with increased metastasis. Further study will continue to identify the true target genes of SMAD6 in pancreatic cancer. In addition, experiments to study the behavioral effects of blocking the DNA-binding capacity of SMAD6 are planned.

In addition, the mechanism by which SMAD6 enters the nucleus remains unknown. An analysis of the protein sequence of SMAD6 yielded no predicted Nuclear Import Sequences (data not shown), suggesting that a binding partner facilitates its entry into the nucleus. Previous work has indicated that SMAD1 was necessary for SMAD6 to bind to the *ID1* promoter (113). Therefore, it is possible the BMP-specific R-SMADs act to shuttle SMAD6 into the nucleus, given that SMAD6 is known to competitively bind these molecules (75). While SMAD6 has been shown to interact with phosphorylated SMAD1, it remains to be seen if native SMAD1 is able to bind to SMAD6, as well, given that SMAD1 has been shown to shuttle between the nucleus and cytoplasm in the absence of BMP stimulation (75,198). Further experimentation will, hopefully, shed light on these aspects of the nuclear biology of SMAD6 nuclear import.

Finally, while we have associated the differential expression of SMAD6 with cell behavior changes *in vitro* and an increase in metastasis in patients, the underlying processes through which these changes are occurring are still unknown. Jeon *et al.*

performed a microarray analysis of non-small cell lung cancer cells after SMAD6 knockdown; Ingenuity Pathway Analysis indicated a number of functional categories that were significantly changed upon SMAD6 modulation, including cell cycle, cell morphology, cellular movement, and cancer (111). We hope to perform a similar experiment to assess the gene expression profiles with and without SMAD6 in pancreatic cancer cells in order to better understand the dynamics of SMAD6 expression in this context.

To sum, we have identified SMAD6 overexpression as a novel factor associated with pancreatic cancer metastasis. More work is certainly necessary to understand the true effects of SMAD6 dysregulation on pancreatic cancer cells. However, it is our hope that this new cancer-associated protein may yield more insight into the biology and therapy of this terrible disease.

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CURRICULUM VITAE

JACQUELINE A. BROSNAN-CASHMAN

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EDUCATION

Johns Hopkins University School of Medicine, Baltimore, MD
Ph.D. Candidate in Pathobiology **(anticipated) 5/ 2014**

Bowdoin College, Brunswick, ME
B.A. in Biology with Honors **5/2008**
Honors Thesis: "Developing RNA Interference Techniques in the Cricket, *Gryllus Bimaculatus"*

AWARDS

- Outstanding Student Research Presentation, Pathobiology Annual Retreat, 2013
- Award for Excellence in Translational Research, Pathology Young Investigators' Day, 2013
- Award for Excellence in Translational Research, Pathology Young Investigators' Day, 2012
- Copeland-Gross Biology Prize (awarded to the graduating senior who has best exemplified the idea of a liberal education during the major program in biology), 2008
- Senior Honors Thesis for the Bowdoin College Biology Department, 2008
- Sarah and James Bowdoin Scholar, 2006-2007
- Idea Network of Biomedical Research Excellence (INBRE) Mentored Fellowship, 2007
- Alvan T.-Viola D. Fuller Fellowship (from American Cancer Society), 2006

RESEARCH EXPERIENCE

Johns Hopkins Medical Institutions, Baltimore, MD **6/2009-present**
Rotation Student and Dissertation Candidate for Christine Iacobuzio-Donahue, M.D., Ph.D.

Hypothesis: Increased expression of endogenous TGF-β and BMP inhibitors will phenocopy loss of SMAD4 in pancreatic cancers

- Generated isogenic cell lines of pancreatic cancer cells with and without SMAD4
- Performed cell behavior assays in pancreatic cells isogenic for SMAD4
- Performed a luciferase screen to determine levels of TGF-β and BMP signaling in pancreatic cancer cell lines
- Performed cell behavior assays in pancreatic cancer cells in which SMAD6 was modulated via overexpression or knockdown
- Performed immunohistochemistry for SMAD6 in pancreatic cancer samples
- Performed ChIP-seq and RNA-seq to detect SMAD6 target genes
- Generated isogenic cell lines of pancreatic cancer cells with and without SMAD4

Johns Hopkins Medical Institutions, Baltimore, MD **1/2009–5/2009**
Rotation Student for Dr. James Herman, M.D.
Hypothesis: The degree to which HDAC2 is enriched on the *Apaf-1* promoter correlates with the success of HDAC inhibitor treatment

- Performed ChIP to assay for the presence of HDAC2 on the *Apaf-1* promoter in leukemia and lymphoma cell lines
- Quantitated ChIP results by quantitative real-time PCR

Johns Hopkins Medical Institutions, Baltimore, MD **10/2008 – 12/2008**
Rotation Student for Dr. David Berman, M.D.,Ph.D.
Hypothesis: The retention of androgen receptor signaling leads to increased proliferation in prostate cancer cells

- Performed immunofluorescence against Ki67 and PSA on prostate cancer xenografts

Bowdoin College, Brunswick, ME **6/2007 – 5/2008**
Research Fellow for Dr. Hadley Horch, Ph.D.
Hypothesis: Semaphorin2a functions as a repellant factor for outgrowing neurites in the cricket brain

- Worked to develop RNA Interference against *semaphorin 2a (sema2a*), a gene expressed in the nervous system of the cricket *Gryllus bimaculatus*
- Designed dsRNA to inject into crickets
- Analyzed expression patterns of *sema2a* mRNA post-dsRNA injection by *in situ* hybridization
- Analyzed expression levels of *sema2a* mRNA post-dsRNA injection by quantitative real-time PCR

Beth Israel Deaconess Medical Center/American Cancer Society, Boston, MA **6/2006 – 8/2006**
Research Fellow for Dr. Sandra Gaston, Ph.D.

Hypothesis: PCR to identify the transcript of the *TMPRSS2:ERG* fusion gene can distinguish prostate cancer in tissue print micropeels from prostate biopsies

- Performed PCR to amplify cDNA from prostate biopsies to identify potential biomarkers, focusing on a translocation between two genes on human chromosome 21: *ERG* and *TMPRSS2*

University of Massachusetts, Lowell, Lowell, MA **7/2005 - 8/2005**
Research Assistant with Dr. Susan Braunhut, Ph.D.

Hypothesis: Catechin compounds suppress the proliferation of breast cancer cells *in vitro*

- Performed cellular proliferation assays on MCF7 and MDA cells in response to potential treatment substances
- Maintained cell cultures of the above cell lines

RESEARCH SKILLS

- Histologic sampling and processing of tissues obtained from rapid autopsy
- Nucleic acid extraction: DNA and RNA
- cDNA synthesis
- Primer design
- Quantitative real-time PCR
- Sanger sequencing and analysis
- Plasmid cloning
- *In situ* hybridization
- Tissue sectioning (cryosectioning and paraffin sectioning)
- Immunohistochemistry
- Immunofluorescence
- Immunoblotting
- Subcellular fractionation
- Cell culture
- Transfection (transient and stable)
- Cell proliferation assay
- Apoptosis assay
- Boyden chamber migration and invasion assays
- Chromatin immunoprecipitation
- Luciferase assays

ABSTRACTS

"SMAD6 upregulation provides an alternative mechanism for BMP inactivation in SMAD4 wild type pancreatic cancers." Johns Hopkins Pathology Young Investigators' Day, April, 2013. Baltimore, MD.

"SMAD6 upregulation provides an alternative mechanism for BMP inactivation in SMAD4 wild type pancreatic cancers." American Association for Cancer Research Annual Meeting, April, 2013. Washington, D.C.

"SMAD6 correlates with metastasis in pancreatic cancer." Johns Hopkins Pathology Young Investigators' Day, April, 2012. Baltimore, MD.

"DPC4 loss results in the activation of alternative oncogenic pathways in pancreatic cancer." Johns Hopkins Pathology Young Investigators' Day, April, 2012. Baltimore, MD.

"DPC4 loss results in the activation of alternative oncogenic pathways in pancreatic cancer." American Association for Cancer Research Annual Meeting, April, 2012. Chicago, IL.

"BAMBI is overexpressed in metastatic pancreatic cancers with genetically intact TGF-β pathways: a potential novel mechanism to escape TGF-β signaling during metastasis formation." Johns Hopkins Pathology Young Investigators' Day, April, 2011. Baltimore, MD.

"BAMBI is overexpressed in metastatic pancreatic cancers with genetically intact TGF-β pathways: a potential novel mechanism to escape TGF-β signaling during metastasis formation." American Association for Cancer Research Annual Meeting, April, 2011. Orlando, FL.

"TGF-β signaling in pancreatic cancer metastasis." Johns Hopkins Pathology Young Investigators' Day, April, 2010. Baltimore, MD.

"Double-stranded RNA-mediated knockdown of semaphorin in the central nervous system of the cricket, *Gryllus bimaculatus*." Society for Neuroscience Annual Meeting. November, 2008. Washington, D.C.

"Developing RNA interference techniques in the brain of the adult cricket, *Gryllus Bimaculatus* ." Society for Neuroscience Annual Meeting. Faculty for Undergraduate Neuroscience (FUN) Poster Session. November, 2007

"Differential gene regulation after auditory interneuron denervation in the cricket *Gryllus bimaculatus*." Society for Neuroscience Annual Meeting. November, 2007. San Diego, CA.

PUBLICATIONS

Makohon-Moore, A., **J.A. Brosnan**, and C.A. Iacobuzio-Donahue. (2013) Pancreatic cancer genomics: insights and opportunities for clinical translation. *Genome Medicine.* **5**(3):26

Yachida, S., C.M. White, Y. Naito, Y. Y. Zhong, **J.A. Brosnan**, A.M. Macgregor-Das, R.A. Morgan, T. Saunders, D.A. Laheru, J.M. Herman, R.H. Hruban, A.P. Klein, S. Jones, V. Velculescu, C.L. Wolfgang, and C.A. Iacobuzio-Donahue. (2012) Clinical significance of the genetic landscape of pancreatic cancer and implications for identification of potential long-term survivors. *Clinical Cancer Research*. **18**(22):6339-6347.

Cui, Y., **J.A. Brosnan**, A.L. Blackford, S. Sur, R.H. Hruban, K.W. Kinzler, B. Vogelstein, A. Maitra, L.A. Diaz, Jr., C.A. Iacobuzio-Donahue, and J.R. Eshleman. (2012) Genetically defined subsets of human pancreatic cancer show unique in vitro chemosensitivity. *Clinical Cancer Research*. **18**(23):6519-6530.

Perez-Mancera, P.A., A.G. Rust, L. van der Weyden, G. Kristiansen, A. Li, A.L. Sarver, K.A.T. Silverstsein, R. Grutzmann, D. Aust, P. Rummele, T. Knosel, C. Herd, D. Stemple, R. Kettleborough, **J.A. Brosnan**, A. Li, R. Morgan, S. Knight, J. Yu, S. Stegeman, L.S. Collier, J.J. ten Hoeve, J. de Ridder, A.P. Klein, M. Goggins, R.H. Hruban, D.K. Chang, A.V. Biankin, S.M. Grimmond, APGI, L.F.A. Wessels, S.A. Wood, C.A. Iacobuzio-Donahue, C. Pilarsky, D. Largaespada, D.J. Adams, and D.A. Tuveson. (2012) The deubiquitinase *USP9X* suppresses pancreatic ductal adenocarcinoma. *Nature*. **486**(7402):266-270.

Duesberg, P., C. Iacobuzio-Donahue, **J.A. Brosnan**, A. McCormack, D. Mandrioli, and L. Chen. (2012) Origin of metastases: subspecies of cancer generated by intrinsic karyotypic variations. *Cell Cycle*. **11**(6): 1151-1166.

Brosnan, J.A. and C.A. Iacobuzio-Donahue. (2012) A new branch on the tree: Next-generation sequencing in the study of cancer evolution. *Seminars in Cell and Developmental Biology*. **23**(2):237-242.

TEACHING EXPERIENCE

Teaching Assistant – Introduction to Biomedical Science (Johns Hopkins University Bloomberg School of Public Health), Summer 2012

Assisted students with questions regarding course material; presented a lecture on the Gastrointestinal System; graded quizzes

Student – Teaching at the University Level (Johns Hopkins University Bloomberg School of Public Health), Spring 2012

Coursework included the development of a teaching philosophy while developing a syllabus for a university course

Teaching Assistant – Pathobiology of Cancer (Johns Hopkins University School of Medicine), Spring 2011

Contacted faculty presenters to remind them of their lecture responsibilities and obtained lecture notes for students; proctored final exam

MEMBERSHIPS

American Association for Cancer Research
Women in Cancer Research
American Association for the Advancement of Science