Author

Having lost her mother to cancer, Zhanna Kovaleva is passionate about cancer research and helping cancer patients. She has been investigating pancreatic cancer for the past two years and, through her mentor, has learned about different aspects of the disease that she may not have studied in her classes. Zhanna encourages students to start thinking about research early and to be aware that it is an immense time commitment. In the future, she plans on becoming a medical oncologist, while continuing to engage in research. Zhanna is a member of the Russian and Hillel clubs and volunteers at the Hollywood Sunset Free Clinic. In 2004, she will be the Head Coordinator for UCI's Biology of Cancer course.

Key Terms

- BxPC3 Pancreatic Cancer Cell Line
- Pancreatic Ductal Adenocarcinoma (PDAC)
- Protein Purification
- Smad4 Mutations
- Stable Transfection
- Transforming Growth Factor Beta (TGF-β)
- TGF-β Receptor (sTβRII)

Approaches to Inhibit and Neutralize Tumor Growth Due to TGF-β1 Produced by Pancreatic Cancer Cells

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Abstract

The increased biological aggressiveness in pancreatic ductal adenocarcinoma (PDAC) is due to numerous molecular alterations, including the presence of Smad4 mutations and overexpression of transforming growth factor beta (TGF-β) isoforms. There is strong evidence that the overexpression of TGF-βs is associated with decreased patient survival. This study examined the possibility of abrogating the TGF-β-Smad4 pathway. BxPC3 clones expressing wild-type Smad4 were established using the Smad4-null BxPC3 pancreatic cancer cell line. Then using techniques such as immunoblotting, Northern blot analysis, and nude mouse tumor formation assays, significant inhibition of tumor growth was shown in BxPC3 cells expressing Smad4 clones compared to BxPC3 cells devoid of Smad4. As a second approach to testing the hypothesis, soluble type II TGF-β receptors (sTβRII) were purified and unfolded using affinity chromatography, and the biological activity of the sTβRII was measured by performing a colometric assay. The data showed that purified sTβRII has the ability to neutralize TGF-β1 actions *in vitro*. Therefore, it is believed that restoring Smad4 functions and using sTβRII will lead to the suppression of pancreatic cancer cell growth *in vivo*, and that these approaches will lead to the development of novel therapeutic strategies in PDAC.

Faculty Mentor

Pancreatic cancers overexpress multiple growth factors, including transforming growth factor-beta (TGF-β) isoforms. This cancer is resistant to TGF-β-mediated growth inhibition because the cells harbor Smad4 mutations, overexpress Smad6 or Smad7, and under-express the type I TGF-β receptor, resulting in loss of negative growth constraints. Ms. Kovaleva, working with her colleagues, demonstrated that it is possible to fix this "broken brake," raising the prospect for novel therapeutic interventions. It was sheer pleasure to work with her, guide her, see her knowledge of cancer biology and the scientific process grow and mature. Her dedication and motivation reinforced my own desire to do more, underscoring the two-way street of beneficial interactions between mentor and student. Thank you Zhanna!

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is responsible for more than 20% of the deaths attributed to gastrointestinal malignancies, making it the fourth most common cause of cancer-related deaths in industrialized countries (Korc, 2003). Previous manuscripts have reported that overexpression of TGF-β proteins in patients presenting PDAC is associated with increased mortality (Friess et al., 1993). The TGF-β family of polypeptides is a major negative regulator of the proliferation of epithelial cell types. Their activity includes regulation of cell growth, differentiation, morphogenesis, and apoptosis (Gold, 1999). TGF-β signals through TGF-β type I (TβRI) and II (TβRII) receptors, which are serine/threonine kinase receptors. Typically, ligands bind to and activate a TβRII homodimer, which recruits, phosphorylates and activates a TβRI homodimer, forming an active tetrameric receptor complex. Phosphorylation of TβRI within the complex triggers the activation of receptor-activated Smads (Smad 2 and Smad 3), that together with a common mediator Smad (Smad 4) form a complex that regulates transcription in the nucleus (Massague and Wotton, 2000). Thus, Smad proteins are a family of transcription factors that play an important role in relaying signals from cell-surface receptors to the nucleus (Heldin et al., 1997).

It has been shown that increases in the biological aggressiveness of pancreatic cancer cells are due, in part, to the increased production of TGF-βs by these cells, and to the presence of Smad4 mutations. However, until recently, the notion that TGF-βs promote pancreatic tumor growth *in vivo* was not convincingly established (Korc, 1998). Two approaches were pursued to test the hypothesis that TGF-βs contribute to the growth advantage of pancreatic cancer cells, and whether this growth can be inhibited. First, BxPC3 clones expressing wild-type Smad4 were established. The control clone (BxPC3 Sham) showed increased tumor growth, whereas analysis of Smad4-expressing BxPC3 clones revealed marked decreases in tumor growth. Second, cell derived TGF-βs were sequestered by soluble TβRII (sTβRII) receptor proteins. It has been demonstrated that sTβRII suppresses the growth of COLO-357 and PANC-1 human pancreatic cancer cells in a nude mouse model (Rowland-Goldsmith, 2001). Thus a study to express sTβRII recombinant protein for use on *in vitro* studies was conducted. It was hypothesized that addition of sTβRII would neutralize the biological activity of TGF-β1, which promotes tumor growth. To test this hypothesis, purification and unfolding of TβRII was performed using affinity chromatography and the biological activity of the sTβRII

was evaluated by monitoring its effects on TGF-β-mediated growth inhibition. Cell growth was determined using the 3-(4,5-methythiazol-2-yl)-2,5-diaphenyltetrazolium bromide (MTT) colometric assay. The colometric assay results showed that purified sTβRII has the ability to neutralize TGF-β1 *in vitro* and ultimately reduce the biological aggressiveness of pancreatic cancer growth.

Together, the two approaches indicate that tumor growth is promoted due to TGF-βs produced by pancreatic cancer cells, which exhibit resistance to TGF-β-mediated growth inhibition *in vivo* because of the presence of Smad4 mutations. In addition, pancreatic cell responsiveness is neutralized by adding purified sTβRII, due to its ability to sequester TGF-βs *in vitro*. These observations suggest a novel therapeutic approach for the treatment of PDAC.

Materials and Methods

Cell Culture

Both COLO-357 and BxPC3 cells were cultured in a 37 °C incubator, humidified with air containing 5% CO_2. The COLO-357 cells were grown in DME (Dulbecco's Modified Eagle's) medium, whereas the BxPC3 cells were grown in RPMI (Roswell Park Memorial Institute) medium. Both media were supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml).

Cell Growth Assays

Cell growth was determined by MTT colometric assay. COLO-357 cells were plated overnight at a density of 7,000 cells per well in a 96-well plate containing DME medium and 10% FBS. The cells were washed with 1x HBSS (Hank's Balanced Salt Solution) and incubated in serum-free medium (DME medium) containing 0.1% BSA, 5 µg/ml transferrin, 5 ng/ml sodium selenite and antibiotics) for 18 hr. The purified and refolded sTβRII was added to the wells in the absence of TGF-β1 for 72 hr for control purposes, and in the presence of TGF-β1 (0.1 nM) in serum-free medium for 72 hr to constitute the experimental group. The assays were initiated by adding 0.625 µg/ml MTT solution and by incubating them for 4 hr. After 4 hr, the dye crystals were dissolved in acidified isopropanol, and the optical density was measured using an optical plate reader.

The data from different trials were collected and expressed as the percentage of non-TGF-β1 treatment control cell growth, and as the means (±SE) of eight determinations per experiment. Statistical analysis was performed using SigmaStat software (Janiel Scientific, San Raphael, CA).

Student's t-test was used with p < 0.01 as the level of significance.

Transfection

The BxPC3 pancreatic cancer cell line was transfected with a wild-type Smad4 construct, previously subcloned into the pCDNA3.1 expression vector carrying a neomycin resistance gene (Invitrogen Life Technologies, Carlsbad, CA). For the control clone (Sham), BxPC3 cells were transfected with an empty plasmid containing neomycin resistance gene. The cells were transfected with these expression constructs using lipofectmine, with 30 μl lipofectamine reagent (Life Technologies, Gaithersburg, MD) and 10 μg of the appropriate plasmid to create the clones, or empty vector alone, for the Sham. After transfection was accomplished, cells were grown to confluency in RPMI complete media. Cells were then split with trypsin solution 1:10 into selection medium (complete medium supplemented with 750 μg/ml G418), and single clones were isolated after 2 to 4 weeks. Following expansion of individual clones, cells from each colony were screened for the expression of Smad4 by the Western blot analysis using a monoclonal mouse anti-Smad4 antibody (B-8 Santa Cruz). In addition, the effects of TGF-β1 on cell growth were determined by cell counting using a hemocytometer.

Immunoblot analysis

Cells were cultured as previously described to a confluency of 80%. After the incubation, the cells were washed with 1x PBS (phosphate buffered saline) at 4 °C and lysed in lysis buffer for 20 min. Cell lysates (30 μg/lane) were prepared and subjected to 10% SDS-PAGE, then electrotransferred to polyvinylidene difluoride membrane and blotted with anti-Smad4 antibody. The protein bound antibodies were visualized using Pierce enhanced chemoluminescence substrate (Pierce, Rockford, IL) and exposed to Kodak BiomaxLight film. In addition, to confirm the equal loading of lanes, the membranes were stripped and reprobed with an anti-ERK2 (antibody against extracellular signal regulated kinase 2) antibody.

In vivo Tumorigenecity Assay

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of UCI, under protocol #1998-1298. The effect of Smad4 to tumorigenecity of BxPC3 was assessed by subcutaneously injecting 1.0×10^6 cells per site (one site per mouse) in the right flank region of female athymic nude mice 4 to 6 weeks of age. Tumor growth was monitored by measuring the tumor mass on the animals using a caliper. Tumor volumes were calculated as the product of $\pi/4$ x length x width x height.

Northern Blot Analysis

Total RNA was extracted from the frozen mice tissue (transfected BxPC3 cells and Sham) by using the single-step acid guanidine thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). RNA was size fractionated in a 1.1% agarose gel at 50 V for 2 hr, electrotransferred at 20 V overnight at 4 °C, and UV crosslinked onto nylon membranes. The membranes were hybridized with radioactively labeled human soluble Smad4 cDNA probes. Signals were visualized by exposing the blots to Kodak Biomax MS films at -80 °C. The membrane was then stripped and rehybridized with a mouse 7S cDNA to confirm equal loading (Balmain et al., 1982).

Results

Following transfection of BxPC3 cells with full-length Smad4 cDNA, several immunoblotting procedures were performed. Western blots were performed to check for the presence of Smad4 in Smad4-transfected BxPC3 cells. BxPC3 Clone 8 and Clone 19 revealed two strong bands measuring approximately 63 kDa, whereas the Sham cells did not form any visible bands (Figure 1). Since the size of Smad4 is between 62 to 65 kDa, it was concluded that the wild-type Smad4 construct was successfully transfected into Smad4-null BxPC3 pancreatic cancer cell line. The membrane was reprobed with anti-ERK2 antibody to verify equal loading of the samples.
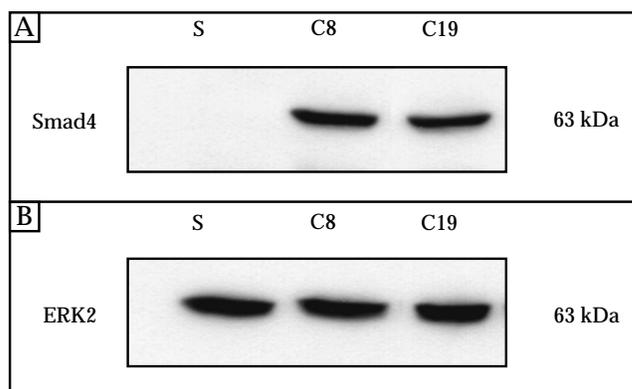

Figure 1
Immunoblotting analysis shows expression of Smad4-transfected BxPC3 cells for BxPC3 Sham (S), BxPC3 Clone 8 (C8), and BxPC3 Clone 19 (C19). (A) Membrane probed with anti-Smad4 antibody. (B) Membrane reprobed with anti-ERK2 antibody to ensure equal loading of lanes.

After obtaining the immunoblotting results, cells were grown in the cell culture and then subcutaneously injected into mice. The pancreatic tumor mass was measured and record-
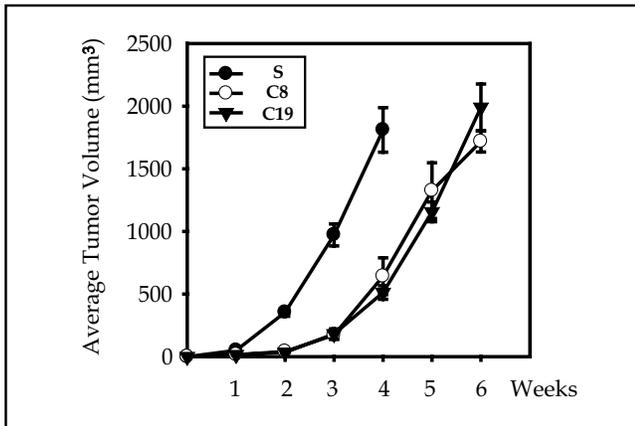
Figure 2

Tumor formation in nude mice. Data are expressed as the means +/– SE from three mice. Representative data were grouped from three independent experiments.
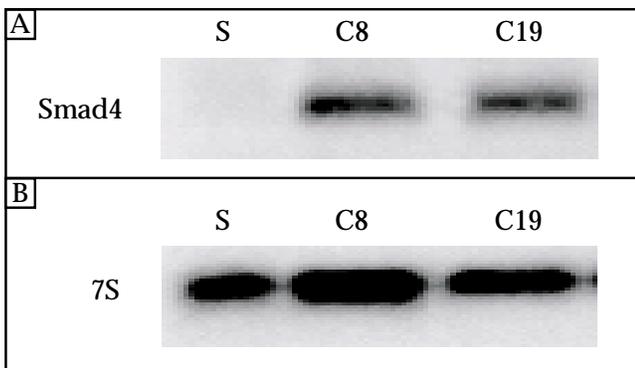

Figure 3

(A) Northern blot analysis of Smad4-expressing BxPC3 clones C8 and C19 and Sham transfected BxPC3 cells (S). (B) The membrane rehybridized with mouse 7S cDNA confirms equal loading of samples.

ed weekly to analyze the tumor growth. The assay revealed a significant inhibition of tumor volume in the BxPC3 clones compared to BxPC3 Sham cells. It also showed that mice lived 50% longer when injected with BxPC3 clones expressing wild-type Smad4, instead of the four-week lifespan expected of mice injected with BxPC3 Sham cells (Figure 2). After tumorigenecity assays were performed, subcutaneous tumors were removed and frozen in liquid nitrogen. RNA was extracted from frozen mice tissue using the guanidine thiocyanate-phenol-chloroform method, and a Northern blot analysis was performed. The Northern blot analysis showed that BxPC3 clones displayed high levels of Smad4 (strong bands), whereas BxPC3 Sham showed no expression of Smad4 (no band). Northern blot analysis confirmed the presence of the Smad4 mRNA construct in the BxPC3
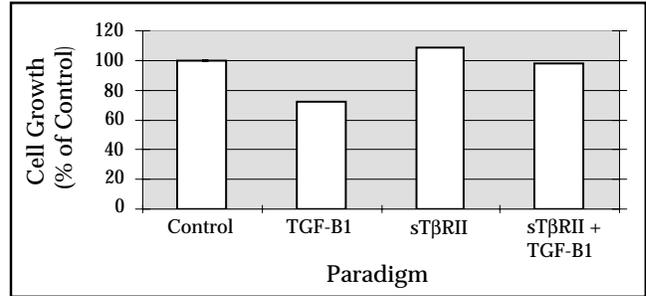

Figure 4

The Neutralizing Activity Assay of purified sTβRII on TGF-β1. COLO-357 cells were seeded in 96-well plates. The cells were placed in serum free medium in the absence of TGF-β1 (control) and the presence of 1 nM of TGF-β1, 2 µg/ml of sTβRII, and 2 µg/ml of sTβRII plus 1 nM of TGF-β1 (sTβRII+TGF-β1) for 72 hr. Growth was determined by MTT colometric assay.

clones and its absence in the BxPC3 Sham. In addition, the membrane was stripped and rehybridized with a mouse 7S cDNA to confirm the equal loading of samples (Figure 3).

During the second study, the responsiveness of purified and unfolded sTβRII protein to TGF-β1 was assessed. The results showed that the addition of TGF-β1 significantly inhibited the growth of COLO-357 cells (with p < 0.01), while the addition of purified sTβRII, 1 µg/ml to COLO-357 cells did not significantly affect the cell growth compared to blank controls (p > 0.1). When the purified sTβRII proteins were mixed with TGF-β1, the cell growth was significantly restored compared to the TGF-β1 only treatments (p < 0.01). These results demonstrate that sTβRII has the ability to neutralize the biological activity of TGF-β1 *in vitro*. The statistical analysis of three consecutive results was performed using the SigmaStat software (Janiel Scientific, San Raphael, CA). A student's t-test was performed, with p < 0.01 taken as the level of significance (Figure 4).

Discussion

PDAC is a deadly disease characterized by numerous cellular alterations, including the overexpression of TGF-β isoforms, perturbations in TGF-β signaling pathways, Smad4 mutations, resistance to chemotherapeutic agents, and enhanced desmoplasia and angiogenesis (Korc, 2003). Previous studies indicated that the overexpression of TGF-β isoforms is associated with decreased patient survival (Friess et al., 1993), suggesting that the abundance of TGF-βs in pancreatic cancer promotes the biological aggressiveness of this disease. It was hypothesized that TGF-βs promote pancreatic tumor growth *in vivo* through

a dual mechanism. First, they act in an autocrine manner to directly stimulate the growth of cancer cells within the tumor micro-environment, but only when Smad4 function is abrogated through mutation and/or deletion. Second, they act in a paracrine manner to promote the proliferation of fibroblasts and endothelial cells, thereby contributing to abnormal epithelial-mesenchymal interactions and aberrant angiogenesis.

To test this hypothesis, two experimental approaches were used. The first approach interfered with the direct growth effects of TGF-βs by restoring normal Smad4 function in BxPC3 cells, which have a homozygous deletion of Smad4. The second approach sought to interfere with the paracrine effects of TGF-βs by sequestering and neutralizing TGF-βs using recombinant soluble type II TGF-β receptor protein.

In the first part of the study, it was shown that it was possible to stably transfect a wild-type Smad4 construct into BxPC3 cells. The growth of transfected clones was not inhibited by TGF-βs to a greater extent than the corresponding sham clones, indicating that growth inhibition *in vitro* was independent of Smad4. However, it was also determined that the expression of transfected wild-type Smad4 was maintained *in vivo*. More importantly, the growth of these clones in nude mice was greatly delayed; indicating that wild-type Smad4 has the capacity to attenuate pancreatic cancer cell tumorigenecity. Although this delay in tumor growth *in vivo* was relatively modest in mice, such a delay is proportional to several years in humans. This observation raises the possibility that the restoration of wild-type Smad4 function may be of therapeutic benefit in pancreatic cancer patients.

The second approach consisted of testing the effects of sTβRII on TGF-β1 actions *in vitro*. It was determined that high-grade and highly purified sTβRII protein could be produced. Moreover, it was demonstrated that purified sTβRII neutralized the biological activity of TGF-β1. Previous studies demonstrated that the expression of constructs encoding sTβRII also neutralized TGF-β1 *in vitro*, and attenuated pancreatic tumor growth and metastasis *in vivo* (Rowland-Goldsmith, 2001, 2002). Therefore, it is thought that sTβRII protein could ultimately be used as a novel therapeutic agent for PDAC patients.

It is not known why TGF-βs promote tumor growth *in vivo*, but inhibit cell growth *in vitro*. Xu and his colleagues at the National Cancer Institute showed that the addition of TGF-β growth factors to cells devoid of Smad4 promotes cell growth (Xu et al., 2000). Moreover, since the addition of

TGF-β1 to cells lacking Smad4 is associated with increased proliferation of gastric polyps, they concluded that Smad4 is a tumor suppressor gene that plays an important role in TGF-β actions. However, another study by Jonson and his colleagues at Lund University suggests that Smad4 may also mediate certain actions independent of TGF-βs (Jonson et al., 2003). Based on the current and previous findings, it is now believed that the inhibition of cell growth *in vitro* is the consequence of the activation of growth inhibitory genes. By contrast, *in vivo*, these genes are under additional influences, and TGF-βs are able to exert direct effects on cancer cells and adjoining cellular elements to promote cancer cell growth.

Conclusion

This study investigated a dual mechanism by which TGF-βs promote pancreatic tumor growth *in vivo*. The results showed that it is possible to stably transfect wild-type Smad4 into pancreatic cancer cells. In addition, expression of transfected wild-type Smad4 was maintained *in vivo*. This led to the conclusion that the restoration of Smad4 function in BxPC3 cells attenuates tumorigenecity and that the restoration of wild-type Smad4 function may be of therapeutic benefit in pancreatic cancer patients. Results obtained from the second approach showed that use of sTβRII *in vitro* leads to suppression of pancreatic cancer cell growth. Therefore, abrogating the growth that derives from aberrant TGF-β dependent signaling may lead to novel therapeutic strategies for treating PDAC.

Further research will be performed to support the data obtained in the current study. Approaches for future investigations include the generation of the orthotopic mouse model and access mechanisms of Smad4 activation. The generation of the orthotopic model is important because it mimics pancreatic cancer in humans and allows for the study of metastasis. In the generation of orthotopic mouse model, tumor tissue derived from subcutaneous injections of the BxPC3 clones will be implanted into the pancreata of other nude mice. This approach allows the study of the effects of the BxPC3 cancer cell lines (expressing wild-type Smad4) in a true pancreatic cancer model and will provide information about the process of metastasis formation *in vivo*. Another way to support and enhance the data would be to access various mechanisms of Smad4 actions. The Korc laboratory is currently examining the effects of Smad4 on the cell cycle, gene expression, and angiogenesis. All in all, this study provides insight into the mechanisms that inhibit and neutralize tumor growth due to TGF-β1 produced by pancreatic cancer cells. Furthermore, abrogating the growth

advantage that arises from the loss of Smad4 may lead to novel therapies for PDAC.

Acknowledgments

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