Ski Promotes Tumor Growth Through Abrogation of Transforming Growth Factor-β Signaling in Pancreatic Cancer

T. Ryan Heider, MD, Suzanne Lyman, MS,* Robert Schoonhoven, MS,‡ and Kevin E. Behrns, MD†*

Objective: We hypothesized that human pancreatic cancer resists TGF-β signaling and cell death through increased Ski expression.

Summary Background Data: Ski is an oncogenic protein that acts as a TGF-β repressor and prevents related gene transcription. Previous work suggests that Ski acts as an oncoprotein in melanoma and esophageal cancer. Ski expression and function have not been determined in human pancreatic cancer.

Methods: Immunohistochemistry and immunoblots assessed Ski expression in human pancreatic cancer. Panc-1 cells were treated with or without Ski siRNA, and Ski and Smad protein expression, transcriptional reporter activation, and growth assays were determined. Panc-1 cells were inoculated in the flank of nude mice and tumor volume and histology assessed after administration of Ski siRNA or control vector.

Results: Ski was abundantly expressed in human pancreatic cancer specimens assessed by immunohistochemistry (91%) and immunoblot analysis (67%). Panc-1 cells exhibited nascent Ski expression that was maximally inhibited 48 hours after transfection with Ski siRNA. TGF-β transcriptional activity was increased 2.5-fold in Ski siRNA-treated cells compared with control ($P < 0.05$). Ski siRNA increased TGF-β-induced Smad2 phosphorylation and p21 expression. Panc-1 growth in culture was decreased 2-fold at 72 hours. A Ski siRNA expression vector injected into nude mice resulted in a 5-fold decrease in growth.

Conclusion: Inhibition of Ski through RNA interference restored TGF-β signaling and growth inhibition in vitro, and decreased tumor growth in vivo.

(*Ann Surg* 2007;246: 61–68)

From the *Department of Surgery, Division of Gastrointestinal Surgery, †Center for Gastrointestinal Biology and Disease, and ‡Center for Environmental Health and Susceptibility, University of North Carolina, Chapel Hill, NC.

Supported by the Department of Health and Human Services, National Institutes of Health (NIDDK; DK 02640-01 and DK 062173-01 to K.E.B.) and (P30ES10126, Center for Environmental Health and Susceptibility to R.S.).

Reprints: Kevin E. Behrns, MD, Department of Surgery, PO Box 100286, Division of General Surgery, University of Florida, 1600 SW Archer Road, Gainesville, FL 32610. E-mail: Kevin.Behrns@surgery.ufl.edu.

ISSN: 0003-4932/07/24601-0061
DOI: 10.1097/SLA.0b013e318070cafa

The nearly equivalent annual incidence and mortality rate for adenocarcinoma of the pancreas provides direct evidence of the difficulty in treating this late-presenting malignancy.[1] Surgical resection remains the only definitive chance at long-term survival, but only 10% to 20% of patients with pancreatic adenocarcinoma present with resectable disease.[2] Therefore, the majority of patients present with advanced local-regional or metastatic disease that is treated by chemotherapy and/or radiation therapy with a potential 3- to 6-month survival benefit and an overall 5-year survival rate of $\leq$5%.[2] Chemoresistance of pancreatic carcinoma is the subject of intense investigation aimed at uncovering mechanisms of resistance to cell death. These efforts, however, have been hindered by lack of a complete understanding of the complex molecular carcinogenesis of pancreatic adenocarcinoma.

Recent basic science investigation has improved our understanding of the molecular genetics of pancreatic cancer in that histologic identification of pancreatic intraepithelial neoplasia (PanIN), precursor lesions to pancreatic carcinoma, has been correlated with genetic alterations such that a progressive model of pancreatic carcinogenesis has been proposed.[3,4] Although a number of molecular changes have been described, the most common mutations occur in the oncogenes *KRAS2* and *ERBB2,* the tumor suppressor genes *p16/CDKN2A, TP53,* and *MADH4 (Smad4),* and the DNA repair gene *BRCA2. KRAS2* mutations are predominant (90%) in pancreatic cancer and appear to occur early in the development of precursors lesions whereas loss of tumor suppressor function from *p16/CDKN2A* inactivation occurs somewhat less frequently (>80%) and later in the sequence of oncogenesis.[3–7] Loss of *TP53* and *MADH4* function are late events in pancreatic carcinogenesis and occur in approximately 70%[8] and 55%[3] of pancreatic cancers, respectively. Loss of *MADH4* function, herein referred to as Smad4, impairs signaling of the growth-inhibitory transforming growth factor beta (TGF-β) and bone morphogenetic protein signal transduction pathways by inhibiting DNA binding activity of Smad pathway mediators to Smad binding elements in target growth-control genes.

TGF-β is a multifunctional cytokine that possesses a variety of important antitumor effects, including the induction of apoptosis, cell cycle arrest, inhibition of angiogenesis, and tumor surveillance. The TGF-β signal is conveyed to the nucleus by binding of the TGF-β ligand to its receptors, phosphorylation of Smads2/3, heterotrimeric assembly of

Smads2/3 with Smad4, and subsequent migration to the nucleus with induction into gene transcription.[8,9] In addition to mutations in Smad4 in pancreatic cancer, downregulation and/or mutation of the type II TGF-β receptor has been described.[11,12] Furthermore, inactivation of p53 may influence TGF-β signaling since p53 has been recently identified as an essential cofactor for effective Smad signaling.[13] Additionally, in vitro studies in a Smad4-null cell line have shown that TGF-β growth inhibitory responses do not require Smad4 expression, suggesting that TGF-β mediated effects can be conveyed through multiple growth control pathways.[14] Collectively, these findings suggest that the molecular pathways in pancreatic carcinogenesis are complex and may interact extensively to produce phenotypic growth characteristics.

To add to the complexity of TGF-β signaling, recent evidence has shown that expression of the oncoprotein, Ski, is evident in several human malignancies. The magnitude of expression is correlated with tumor progression in melanoma,[15] esophageal,[16] and colorectal cancers.[17] Ski is a proto-oncogene that negatively regulates TGF-β signaling and is capable of transforming and inducing anchorage-independent growth of chicken embryo fibroblasts.[18] Ski physically binds to the common elements Smad2 and 3 but has also been shown to inhibit TGF-β signaling through inhibition of Smad2 phosphorylation,[19] cytoplasmic sequestration of Smad complexes,[20] and prevention of the Smad 2/3/4 complex formation with essential nuclear cofactors.[21] However, Ski's role in abrogating TGF-β signaling and inhibiting tumor growth in pancreatic cancer is unknown. Therefore, the aim of this study was to determine if Ski is expressed in human pancreatic cancer and to examine mechanisms by which Ski may regulate pancreatic cancer growth responses to TGF-β.

MATERIALS AND METHODS

Reagents and Antibodies

The following materials were purchased from the manufacturer: mouse monoclonal anti Cip-1/WAF-1/p21 (Pharmingen, San Diego, CA), mouse monoclonal anti-β-actin AC-15 (Sigma), goat polyclonal anti-Smad 2/3 (N-19) (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal antiphospho-Smad 2 (Ser465/467) (Cell Signaling, Beverly, MA), rabbit polyclonal anti-Ski (Upstate, Lake Placid, NY), peroxidase-conjugated anti-rabbit, antimouse and anti-goat IgG, R-phycoerythrin-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA), and recombinant human TGF-β1 (R&D Systems, Minneapolis, MN). The p3TP-luciferase construct with 3 TGF-β-responsive elements, a plasminogen activator inhibitor-1 (PAI-1) promoter,[22] and an upstream adenoviral E4 promoter was a gift from Dr. Richard A. Rippe (Chapel Hill, NC). Animal use and care was approved by the University of North Carolina Institutional Animal Care and Use Committee.

Human Tissue Specimens

Tumor specimens from 20 total patients who had undergone resection for pancreatic adenocarcinoma were analyzed for Ski expression by immunohistochemistry or immunoblot. Pancreatic tissue from 5 patients with chronic pancreatitis and 9 normal pancreatic specimens were used for comparative purposes.

Immunoblot Analysis

For whole cell extracts, cells were rinsed twice in PBS and lysed in buffer containing 0.05 mol/L Tris, pH 7.3, 0.15 mol/L NaCl, 1% NP40, 0.5% deoxycholate, for 10 minutes at 4°C. Samples were centrifuged at 14,000 rpm to remove debris and the protein concentration was determined by Bradford assay. After SDS-polyacrylamide gel electrophoresis, samples were transferred to PVDF membranes and blocked in 5% nonfat milk in TBS-T. After incubation with rabbit polyclonal anti-Ski antibody, the antibody was diluted 1:1000 in blocking buffer for 1 hour, blots were washed for 15 minutes in TBS-T, incubated with HRP-conjugated secondary antibody (1:1000) for 30 minutes and visualized by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

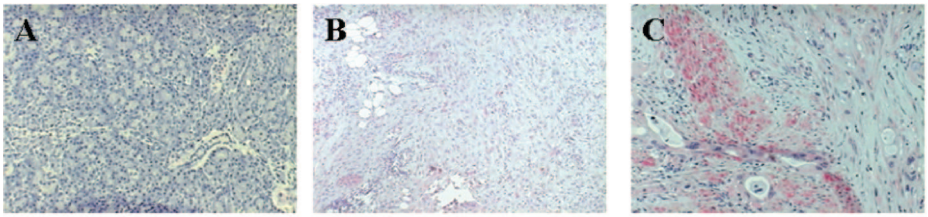
Cellular extracts were obtained by lysis in buffer containing 10 mmol/L HEPES, pH 7.9, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.1% Triton X-100, 0.5 mmol/L DTT, and 0.5 mmol/L protease and phosphatase inhibitor cocktails (Sigma Aldrich, St. Louis, MO). The cytoplasmic fraction was collected and the nuclear pellet was resuspended in a buffer containing 20 mmol/L HEPES, pH 7.9, 1.5 mmol/L $MgCl_2$, 0.42 M NaCl, 0.2 mmol/L EDTA, 1% NP40, 25% glycerol, 0.5 mmol/L DTT, and 0.5 mmol/L protease inhibitor cocktail.

Immunohistochemistry

Sections (5 μm) of formalin-fixed samples were deparaffinized with xylene and rehydrated through graded alcohol into distilled water. Sections were subjected to heat-induced epitope retrieval in target unmasking fluid (Zymed Laboratories, Inc., San Fransisco, CA) prior to staining. Sections were rinsed in PBS with 1% Tween 3 times, incubated with primary antibody (1:100 in 1% BSA) for 30 minutes, rinsed 3 times, and incubated with secondary antibody for 30 minutes. Endogenous phosphatase activity was blocked with 5 mmol/L levamisole, and the alkaline-phosphatase based chromogen was applied as directed by the manufacturer (New Fuchsin, BioGenex, San Ramon, CA). Sections were counterstained with hematoxylin, mounted, and visualized via light microscopy. Antibody staining was quantified by digital image analysis (BioQuant Image Analysis Corp., Nashville, TN).

Transcriptional Reporter Assay

Cells were seeded at a density of 2×10^5 in 6-well plates the day prior to sequential transfection with 5 μg of siRNA using RNAiFect (Qiagen Inc., Valencia, CA) and 4 μg of p3TP-luciferase DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 24 hours of treatment, cells were harvested for luciferase expression using the Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI) according to manufacturer's instructions and analyzed using a Monolight 2010 Luminometer (Analytical Luminescence Laboratory, San Diego, CA).



Tissue	N	Detection of Ski (#)	μ/HPF
Normal	9	0	0
Chronic pancreatitis	5	4	0.028
Adenocarcinoma	11	10	11.81

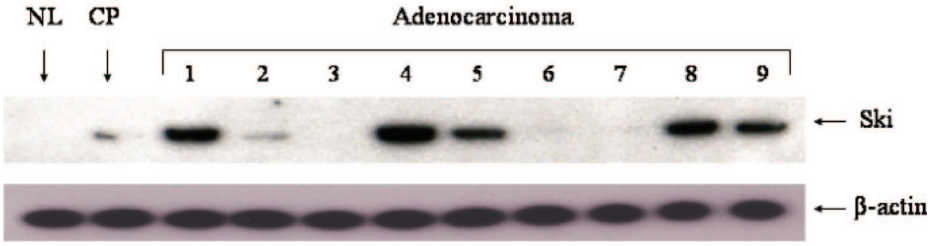


FIGURE 1. Expression of Ski in human pancreatic cancer specimens. Normal pancreas (A) and chronic pancreatitis specimens (B) demonstrate no or minimal Ski expression, respectively, when compared with pancreatic adenocarcinoma (C) that has diffuse background and intense focal staining. Computer-assisted digital quantification of Ski staining is presented in the table. Pancreatic cancer specimens demonstrated significantly ($P < 0.05$) more Ski expression than normal pancreas and chronic pancreatitis. B, Western blot analysis of 9 human pancreatic adenocarcinoma specimens demonstrates staining for Ski in 5 of 9 samples compared with representative samples of normal (NL) pancreas and chronic pancreatitis (CP).

Cell Culture and siRNA Transfection

Panc-1 cells were obtained from the American Type Tissue Collection (Manassas, VA) and maintained in Dulbecco Modified Eagle Medium with 4mmol/L L-glutamine and adjusted to contain 4.5 g/L glucose and 1.5 g/L sodium bicarbonate, supplemented with 10% fetal bovine serum, and maintained at 37°C in 5% CO_2. Cells were transfected with siRNA using RNAinfect (Qiagen, Germantown, MD) according to manufacturer's instructions for adherent cells. The efficiency of silencing was monitored by immunoblot for Ski 24 to 48 hours after transfection. All transfections were performed with a combination of 2 21-nt double-stranded oligonucleotides targeted against positions 910–929 (AACTGGCGGGCCTACATCCT) and 389–408 (AATTCTCCGAACGTGTCACGT) of the human Ski (DNA gene accession number NM003036) or with control siRNA directed against the sequence AACTGGCGGGCCTACATCCT (Qiagen, Germantown, MD).

In Vivo Tumor Assessment

Five-week-old, male, athymic nude mice (HSD nu^-/nu^-, Harlan-Sprague Dawley, Indianapolis, IN) were maintained in climate controlled conditions with 12-hour light/dark cycles and provided water and standard chow ad libitum. For in vivo siRNA inhibition experiments, 1×10^6 cells were suspended in 100 μL of endotoxin-free PBS and injected subcutaneously into the flank. The 21-nt sequence targeting the 389–408 region of Ski was inserted into a DNA vector containing a RNA polymerase III promoter upstream of the oligonucleotide sequence (Genescript, Piscataway, NJ). Beginning 24 hours after tumor injection, 50 μg of plasmid vector expressing Ski-specific siRNA or control was diluted in 1 mL of endotoxin-free PBS and hydrodynamically injected via tail vein over 5 to 10 seconds 3 times per week as described previously.[23,24] Tumor volume was calculated using the formula $V = \pi/6 \cdot l \cdot w^2$.

Data Analysis

All in vitro experiments were performed in triplicate. For in vivo experiments, a minimum of 5 mice were studied per group. Data are reported as mean ± SEM. Statistical analysis was performed by *t* test or Log Rank Test where appropriate utilizing StatView software (SAS, Cary, NC). A *P* value of less than 0.05 was considered significant.

RESULTS

Human Pancreatic Cancer Expresses Ski Oncoprotein

In total, 11 human pathologically confirmed pancreatic cancer specimens, 9 normal pancreas specimens, and 5 chronic pancreatitis specimens were stained for immunohistochemical detection of Ski expression. Ski was not detected in normal pancreas (Fig. 1A).

However, in pancreatic cancer specimens, Ski expression was increased markedly compared with normal and chronic pancreatitis specimens and could be detected in the cytoplasm and the nucleus of epithelial cells (Fig. 1A, C). Chronic pancreatitis samples exhibited diffuse, weak staining pattern that was only slightly more prominent than normal pancreas specimens (Fig. 1A, B). Ski expression was in-

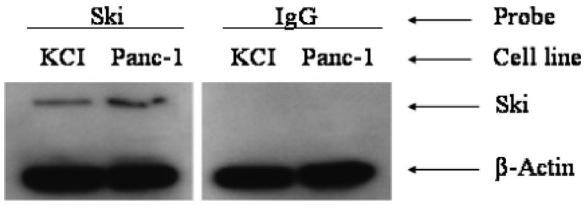


FIGURE 2. Detection of Ski in vitro. Ski is endogenously expressed in both the Panc-1 and KCI-Moh1 cell lines.

creased markedly in pancreatic cancer compared with chronic pancreatitis and normal pancreas specimens (Fig. 1A, table).

In addition, Ski expression was examined by immunoblot analysis of frozen tissue samples from normal pancreas (n = 9), chronic pancreatitis (n = 5), and pancreatic cancer (n = 9) resection specimens that originated from different tumor sources than formalin-fixed specimens (Fig. 1B). Significant expression of Ski was detected in 5 of 9 pancreatic cancer specimens, but not in normal pancreas or chronic pancreatitis specimens.

Ski Expression in Pancreatic Cancer Cell Lines

The human pancreatic cancer cell line Panc-1 was examined for expression of Ski by immunoblot analysis (Fig. 2). Although the KCI-Moh1 cell line also expressed Ski, the Panc-1 cell line was chosen for further in vitro studies because of strong Ski expression and known expression of wild-type Smad4 protein.[25]

siRNA Targeted Against Ski Effectively Decreases Ski Protein

Commercially available 21-nt sequences targeting Ski were obtained, and the oligonucleotide sequence directed against the 389–409 region of Ski was effective in gene silencing in Panc-1 cells (data not shown). When controlled for beta-actin loading, Ski expression was decreased 49.7% at 24 hours and was maximally decreased (71.4%) at 48 hours (Fig. 3, lanes 2 and 3, respectively) compared with control siRNA. Ski expression returned to baseline by 72 hours after transfection (data not shown). These findings are consistent with the transient nature of transfection-mediated siRNA gene silencing. Transfection with control siRNA (Fig. 3, lanes 4 and 5) and with scrambled, nonsilencing siRNA (data not shown) did not decrease Ski protein expression.

Inhibition of Ski Permits TGF-β Signal Transduction

Panc-1 cells transfected with Ski siRNA (Fig. 4, right panel), control siRNA (Fig. 4, middle panel), or no transfection (Fig. 4, left panel) were treated with TGF-β and probed for phosphorylated Smad2. Panc-1 cells were treated with TGF-β alone or with TGF-β and control siRNA and exhibited only minimal increases in the phosphorylated form of Smad2. In contrast, inhibition of Ski via siRNA transfection resulted in a rapid and sustained increase in phosphorylated Smad2 in the Panc-1 cells that have nascent expression of Ski (Fig. 4, right panel). This is consistent with functional Ski inhibition of the TGF-β signal transduction pathway in Panc-1 cells.

To determine if the changes in Smad2 phosphorylation seen after Ski inhibition resulted in increased activity at the level of the TGF-β promoter, Panc-1 cells were transfected with the TGF-β responsive p3TP-luciferase construct. Cells were simultaneously transfected with either Ski siRNA or control siRNA and subsequently treated with TGF-β for 24 hours. Cells transfected with control siRNA exhibited no increase in luciferase activity compared with cells transfected with the p3TP-luciferase construct alone. However, siRNA inhibition of Ski resulted in a 2.5-fold increase in luciferase activity at 24 hours (Fig. 5).

Ski Inhibition Relieves TGF-β-Induced p21 Expression

TGF-β can regulate cellular growth control through both apoptotic and cell cycle arrest mechanisms, depending on the cellular target.[26] We examined apoptosis in Panc-1 cells treated with TGF-β for up to 72 hours and found that inhibition of Ski through siRNA did not result in increased apoptosis (data not shown). We examined the expression of p21, a TGF-β-responsive cell cycle inhibitor, in the presence or absence of Ski inhibition (Fig. 6). Following TGF-β stimulation alone or TGF-β treatment after transfection with control siRNA, p21 expression was low. However, cells treated with Ski siRNA prior to TGF-β administration exhibited a marked increase in p21 expression by 6 hours.

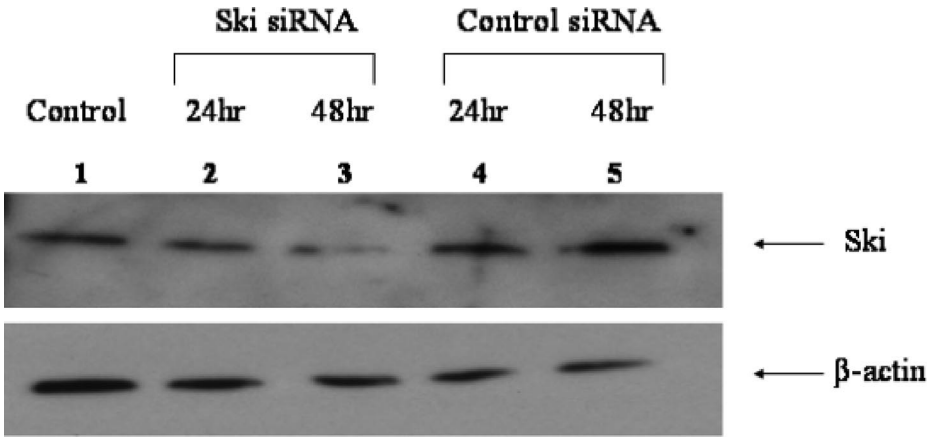


FIGURE 3. Inhibition of Ski by siRNA transfection. Panc-1 cells were transfected with Ski-specific siRNA (lanes 2 and 3) or control siRNA (lanes 4 and 5). Ski expression was knocked down in cells transfected with Ski siRNA at 48 hours (lane 3) but not in cells transfected with control siRNA (lanes 4 and 5).

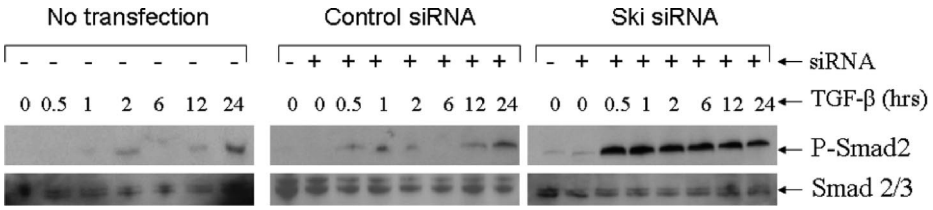


FIGURE 4. Ski inhibition permits TGF-β-dependent phosphorylation of Smad2. Panc-1 cells were treated with Ski siRNA (right panel), control siRNA (middle panel), or no transfection (left panel) for 24 hours prior to the administration of TGF-β (5 ng/mL). Immunoblots demonstrated an increase in phospho-Smad2 expression beginning as early as 30 minutes (right panel) and persisting for 24 hours.

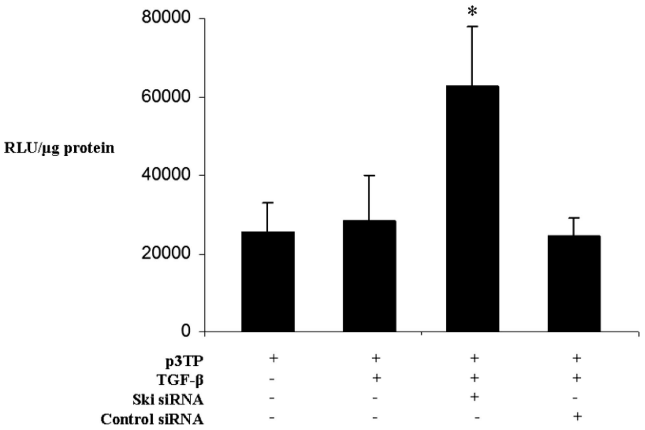


FIGURE 5. Inhibition of Ski increases TGF-β transcriptional reporter activity. Panc-1 cells were transfected with the TGF-β responsive, luciferase reporter, p3TP. TGF-β-induced luciferase activity was measured in Panc-1 cells transfected with Ski or control siRNA. Administration of TGF-β (5 ng/mL) produced no significant increase in luciferase activity over control in these Panc-1 cells with constitutive Ski expression. However, after siRNA inhibition of Ski, TGF-β induced a significant increase in luciferase activity ($*P < 0.05$).

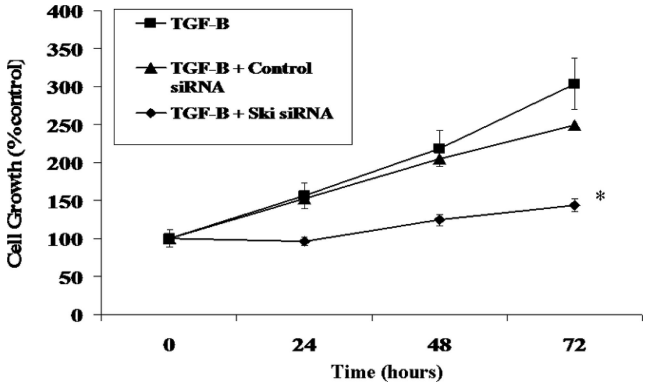


FIGURE 7. Inhibition of Ski augments TGF-β-induced growth inhibition in vitro. Cells were transfected with Ski siRNA or control siRNA or not transfected 24 hours prior to treatment with or without TGF-β (5 ng/mL; time 0). Nontransfected cells and cells transfected with control siRNA demonstrated similar growth curves, with a 2.5- to 3-fold increase in cell number at 72 hours. Cells transfected with Ski siRNA, however, demonstrated only a 50% increase in cell number ($*P < 0.005$).

Inhibition of Ski Decreases Panc-1 Growth Both In Vitro and In Vivo

To determine if TGF-β signaling associated with Ski inhibition resulted in altered cell growth, proliferation of Panc-1 cells was assessed by counting cells over 72 hours in the presence or absence of TGF-β (Fig. 7). Administration of TGF-β resulted in a nonsignificant decrease in growth compared with control (data not shown). However, administration of Ski siRNA prior to TGF-β treatment resulted in a significant decrease in growth at 72 hours when compared with TGF-β treatment alone or with control siRNA (Fig. 7).

Panc-1 cells were injected subcutaneously into the flank of nude mice. Mice were subsequently injected via tail vein 3 times per week for 2 weeks with control vector or the siRNA expression vector pRNA-U6.1-Ski, a DNA vector that contains the 21-nt Ski siRNA sequence downstream from an RNA polymerase III promoter. Tumor size was recorded

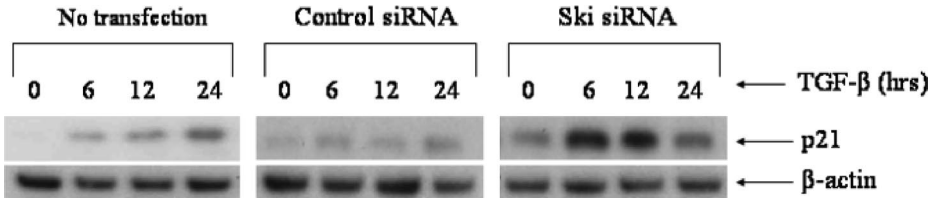


FIGURE 6. TGF-β-induced expression of p21 following Ski inhibition. Nontransfected Panc-1 cells (left panel), those transfected with control siRNA (middle panel), or Ski siRNA after 24 hours (right panel) were treated with TGF-β (5 ng/mL) for 0 to 24 hours. Treatment with TGF-β induced comparable increases in p21 expression in both nontransfected cells (left panel) and cells transfected with control siRNA (middle panel). Transfection with Ski siRNA prior to TGF-β administration (right panel) was associated with an earlier and more sustained increase in p21 expression when compared with nontransfected cells and those transfected with control siRNA.

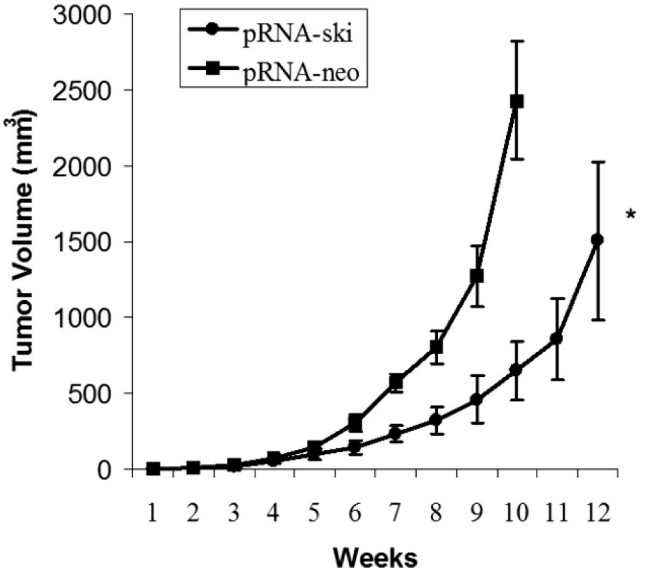


FIGURE 8. Inhibition of Ski decreases tumor growth and enhances survival in vivo. Mice treated with Ski siRNA expression vector demonstrated a 5-fold decrease in tumor volume at 10 weeks (*$P < 0.001$).

prior to each injection (Fig. 8). In vivo delivery of the Ski siRNA decreased tumor volume nearly 5-fold at 10 weeks. Tumor volume was low during the treatment phase and increased gradually during weeks 5 to 8, suggesting a prolonged effect of Ski siRNA. However, by week 9, growth increased dramatically, indicating little growth inhibition from Ski siRNA. Mice in the pRNA-U6.1-Ski group had decreased tumor volume at the time of death compared with control mice (data not shown).

At the conclusion of the observation period, tumors from mice treated with pRNA-U6.1-Ski showed no change in Ski expression compared with tumors in mice treated with PBS or control vector (Fig. 9A, right panel). However, tumors harvested during active treatment with pRNA-U6.1-Ski exhibited decreased Ski expression compared with control (Fig. 9A, left panel, right lane).

After death, sections of representative tumors from each of the treatment groups were stained with hematoxylin and eosin. Tumors from mice treated with pRNA-U6.1-Ski exhibited a marked increase in tumor necrosis compared with mice treated with control vector or PBS (Fig. 9B).

DISCUSSION

Pancreatic cancer is a biologically aggressive, highly metastatic tumor that, at presentation, has frequently spread beyond the limits of curative resection and is refractory to current chemotherapeutic regimens. The molecular characteristics of pancreatic cancer that confer sustained growth, metastatic potential, and chemotherapeutic resistance are unknown. Several molecular genetic defects have been profiled in pancreatic cancer, and alterations in the TGF-β pathway have been among the most prevalent. Defects in TGF-β signal transduction are common in many gastrointestinal tumors, and the oncoprotein Ski has been identified recently as a negative regulator of this pathway and correlated with a less favorable outcome in several human malignancies.[16,17] The aim of this study was to determine if Ski expression occurred in human pancreatic adenocarcinoma and to investigate the significance of this protein in modulating tumor growth. We found that expression of Ski occurred in human pancreatic cancer resection specimens, and inhibition of Ski decreased pancreatic tumor growth both in vitro and in vivo.

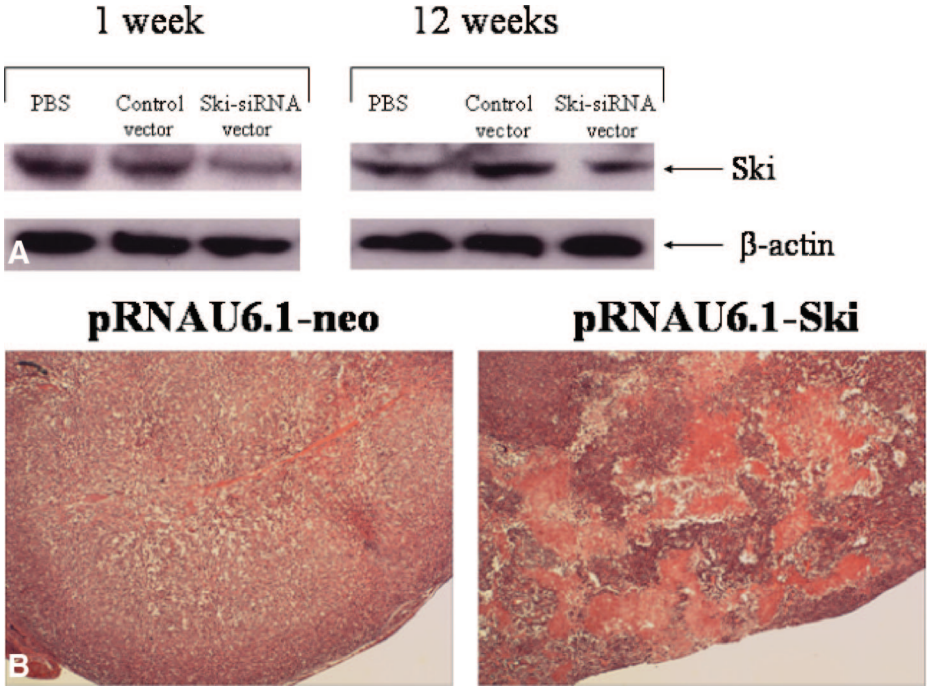


FIGURE 9. Ski siRNA expression vector decreases tumor levels of Ski and is associated with increased tumor necrosis. A, In tumors from mice treated with Ski siRNA expression vector, tissue levels of Ski were decreased when compared with control. At the conclusion of treatment (right panel), no substantial decrease in Ski protein expression in the experimental group was noted. B, Representative sections of tumor taken at the conclusion of treatment from mice treated with control vector (left) or Ski siRNA expression vector (right) were stained with hematoxylin and eosin. Tumor from mice treated with Ski siRNA expression vector demonstrated an increase in tumor necrosis when compared with control samples.

Furthermore, Ski inhibited TGF-β signaling, in part, through inhibition of Smad2 phosphorylation. In addition, inhibition of Ski increased p21 expression in response to TGF-β, thereby implicating impaired cell cycle regulation in Ski-expressing tumors.

The importance of the TGF-β signaling pathway in the progression of pancreatic cancer has been well studied. Several in vitro studies of this pathway have confirmed that attenuation of the TGF-β-induced Smad signaling pathway leads to resistance to growth arrest,[27] and restoration of Smad4 reverses the invasive phenotype of pancreatic adenocarcinoma cells.[28] Additionally, restoration of TGF-β signaling in MIA PaCa-2 cells sensitizes these cells to ionizing radiation.[29] Importantly, recent evidence suggests that an intact TGF-β signal transduction pathway results in improved outcomes in patients with pancreatic cancer. The expression of TGF-β and its downstream effector, p21, has been shown to identify a subset of patients with significantly increased survival compared with those with tumors that do not express either protein.[30] In addition, expression of Smad4 has been associated with improved outcome in patients with pancreatic adenocarcinoma.[31] Ski has been identified in several nonpancreatic human malignancies, and its role in tumor biology and disease progression has been increasingly appreciated. Ski expression was identified in each of 44 human melanoma specimens examined, and a stable transfectant expressing an antisense Ski vector demonstrated increased growth inhibition in the presence of TGF-β when compared with control cells.[15] In colorectal cancer, Ski expression was found in 10% of colorectal tumor biopsies and independently predicted decreased disease-free and overall survival.[32] Moreover, expression of Ski has been found to correlate with depth of invasion and pathologic stage in esophageal cancer.[16] Additionally, in a subset of patients with esophageal cancer and low TGF-β expression, expression of Ski predicted decreased overall survival.[16]

The molecular mechanisms by which Ski promotes tumor growth are not completely understood. Previous work has demonstrated that its biologic actions are mediated through binding a consensus GTCTAGAC sequence of Smads2, 3, and 4.[33] Ski-Smad complex formations may be associated with impaired phosphorylation or cytoplasmic trafficking of Smad proteins. Also, a Ski-Smad complex may trigger proteosomal degradation and loss of the TGF-β-mediated signal.[34] Although nuclear expression of Ski is low, it may function to bind important Smad complex cofactors and inhibit DNA binding and transcriptional activation. Whether the abrogation of the TGF-β signal is largely dependent on inhibition of Smad2 phosphorylation, cytoplasmic sequestration of Smad 2/3/4, interference with nuclear cofactor assembly, or a combination of these mechanisms has not been determined. Alternatively, progression of melanoma has been associated with Ski activation of the Wnt/beta-catenin pathway, which resulted in transcription of microphthalmia-associated transcription factor and Nr-CAM that were associated with melanoma cell growth and increased motility.[35] Importantly, in human melanoma, Ski expression is correlated with cell cycle progression by repressing the activity of protein RB and by decreasing Smad-mediated induction of p21.[36] Given the importance of defects in the TGF-β-induced Smad signaling pathway in human malignancy, a negative regulator of this pathway, such as Ski, could act as functional knockout of the Smad 2/3/4 signaling complex and contribute to disease progression.

These findings suggests that Ski expression occurs frequently in human pancreatic adenocarcinoma and contributes to tumor growth through inhibition of TGF-β signaling both in vitro and in vivo through inhibition of Smad signaling. The inhibition of Smad signaling by Ski may be mediated by inhibition of Smad phosphorylation or transcriptional cofactor assembly. These molecular events may permit changes in cell cycle regulation. This study provides depth to the field of study of TGF-β effects in pancreatic cancer and proof of principle that alterations in the TGF-β growth control pathway can occur through multiple mechanisms. As the science of TGF-β, Smad, and Ski advance in pancreatic adenocarcinoma, potential diagnostic, prognostic, and therapeutic tools are on the horizon.

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