Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States, resulting in 30,000 deaths each year, in part because it is one of the most lethal cancers with a death-to-incidence ratio of 0.99 (1). The lethality of pancreatic cancer is largely due to late stage of diagnosis and resistance to current therapies. More than 80% of patients are unresectable and most present with metastatic disease due to aggressive localized invasion and a high incidence of early metastasis (2). Worldwide, the incidence of death from pancreatic cancer has increased, which, together with the lack of current therapies, emphasizes the need to determine the mechanisms of pancreatic cancer progression and better targets for diagnosis and treatment.

The current model of pancreatic cancer progression includes mutations that activate K-ras, followed by inactivating mutations or loss of expression of tumor suppressor genes including p53, p16^INK4A, and SMAD4 (3). Sonic hedgehog (SHH) and other proteins downstream of the hedgehog pathway were recently detected in precursor lesions and samples of primary tumors from patients with pancreatic adenocarcinoma, which implicates this pathway in the initiation and progression of pancreatic cancer given its absence in normal adult pancreas (4, 5). Transgenic mouse models in which SHH was expressed in vivo known to express SHH and form moderately differentiated tumors in nude mice. Our data provide evidence that expression of SHH contributes to the formation of desmoplaysia in pancreatic cancer, an important component of the tumor microenvironment.

### Materials and Methods

**Cell lines and culture conditions.** The cell line we used for these experiments was hTert-HPNE. The cells were originally isolated from the ductal structure of a human pancreas and immortalized by expressing the catalytic subunit of telomerase (h-Terr), along with subsequent transductions with retroviral constructs to introduce a Kras mutation, the human papillomavirus E6 and E7 peptides, and the SV40 small t antigen, as described previously (7, 8). The hTert-HPNE cells were maintained in Medium D containing 1 volume of M3 base (InCell Corp.), 3 volumes of glucose-free DMEM, 5% fetal bovine serum (FBS), 5.5 mmol/L glucose, 10 ng/mL epidermal growth factor, and 50 μg/mL.
Translational Relevance

The data in this article suggest that SHH contributes in a paracrine manner to the formation of desmoplasia in pancreatic cancer. The robust desmoplastic reaction seen in many pancreatic cancers, which includes fibroblasts, endothelial cells, and other stromal cell types, is believed to contribute to tumor progression by creating protumorigenic effects in the tumor microenvironment. Desmoplasia also creates barriers to the delivery of therapeutic compounds to tumor cells. Inhibition of desmoplasia would inhibit tumor growth and progression, and elimination of desmoplasia would be predicted to enhance the potential of delivery of some therapeutics to pancreatic tumors.

gentamicin. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO2.

For overexpression of SHH, the cDNA of SHH was cloned into the pWZL-blast retroviral vector [courtesy of Dr. Wade Bushman (University of Wisconsin Medical School, Madison, WI) and Dr. Michel Ouellette (Eppley Institute, Omaha, NE)]. Clones expressing SHH were isolated by serial dilutions and selection with blastocidin. A control vector was transduced into the control cell lines.

The Capan-2 pancreatic cancer cell line is maintained in McCoy’s 5A medium and are cultured at 37°C in a humidified atmosphere containing 5% CO2. Pancreatic stellate cells and fibroblasts were isolated from an islet transplant program at the University of Nebraska Medical Center and are maintained in RPMI medium at 37°C in a humidified atmosphere containing 5% CO2.

Western blot analysis. Whole cell lysates were prepared by adding 1.5-mL cell lysis buffer [10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride and 1% Triton X-100] to cells grown to 80% confluence in T175 tissue culture flasks. Sixty micrograms of protein were loaded onto 4% to 20% Novex Tris-glycine gradient denaturing polyacrylamide gels (Invitrogen) in a 1× SDS-PAGE running buffer, containing 1 g/L SDS, 3 g/L Tris-base, and 14.4 g/L glycine. The proteins were transferred onto polyvinylpyrolidine difluoride membranes by electrophoresis. Once transferred, the membranes were blocked in Blotto [5% dry milk resuspended in 1× PBS, and resuspended at a density of 3 × 10^5/30 μL]. The cells were injected between the scapulae.

Invasion assay. The in vitro invasive potential of cells overexpressing SHH was assayed using the Biocoat Matrigel Invasion Chamber (Becton Dickinson Labware). The invasion assay with pancreatic myofibroblasts included an initial seeding of 50,000, 250,000, or 500,000 T-HPNE or T-HPNE.SHH cells in the lower chambers. The cells were allowed to adhere for 8 h. The medium was then removed and replaced with RPMI medium containing 7% FBS. The upper chambers, 250,000 pancreatic myofibroblasts were seeded and then allowed to incubate for 22 h at 37°C and 5% CO2. For invasion with the pancreatic fibroblasts with rhSHH, 250,000 cells were seeded in the upper chamber in RPMI medium containing 7% FBS. The lower chambers included control RPMI with 7% FBS, 0.1 μg/mL recombinant SHH (R&D Systems), and 1.0 μg/mL recombinant SHH. These chambers were also allowed to sit for 22 h at 37°C and 5% CO2. For analyses of all invasion assays, cells on the upper portion of the matrix membrane were wiped off with a cotton-tipped swab and cells on the bottom of this membrane were fixed and stained with Diff-Quick staining kit (Allegiance) and observed by light microscopy. The number of invading cells was quantified by counting the membranes at ×200 magnification.

Subcutaneous injections in athymic nude mice. Three million cells were injected s.c. in athymic nude mice. After injection, the cells were trypsinized, counted, washed twice in 1× PBS, and resuspended at a density of 3 × 10^5/30 μL. The cells were injected between the scapulae. Tumor growth was monitored every 2 d by measuring the diameters with a caliper. The mice were euthanized when the tumor volume reached 1,800 mm^3.

Orthotopic implantation in athymic nude mice. One million T-HPNE and T-HPNE.SHH cells were each injected into the pancreas of 12 or 13 mice, respectively, to analyze tumor desmoplasia in response to overexpression of SHH. Preparation of cells was as described above for the s.c. injection model. Orthotopic implantation of T-HPNE cell lines was done as described previously (9).

SHH inhibition in orthotopic models. To inhibit SHH in the Capan-2 orthotopic models, we administered the 5E1 antibody by i.p. injections at a dosage of 500 μg/mouse once a week for 10 wk. An IgG control antibody, 4E11, was also given to a separate group of mice at a concentration of 500 μg/mouse, once per week.

Confocal and immunohistochemical analyses. To analyze cell morphology in the orthotopic tumors, primary tumors were resected and placed in formalin for 24 h before being fixed for staining. Staining with H&E allowed for the visualization of cells within the tumor sections. Fibroblasts were visually differentiated from tumor cells on the basis of cellular morphology and by staining with the markers described below.

To identify SHH in the primary human tumor sections, rabbit anti-SHH primary antibody (1:200; Santa Cruz) was incubated on paraffin-embedded tissue sections for 2 h at 37°C. The secondary antibody (antirabbit, 1:1,000) was incubated for 50 min at 37°C.

Statistical analysis. The in vitro wound healing assays, and invasion assays were analyzed with one-way ANOVA test as part of the Prism statistical analysis package. Significance was determined at the 95% confidence interval.

Results

Inhibition of SHH signaling decreases desmoplasia. We evaluated the contribution of SHH to the growth properties of pancreatic tumor cells by administering monoclonal antibody
5E1 (11), a blocking antibody to SHH, to mice challenged orthotopically with Capan-2, which expresses moderately high levels of SHH and forms moderately differentiated pancreatic tumors with desmoplasia (12). One million Capan-2 cells were implanted orthotopically into the pancreas of athymic nude mice and 500 μg of 5E1 were administered once per week. An isotype control IgG antibody, 4E11, was given to a control group of mice. Treatment with 5E1 resulted in a decreased tumor volume from an average of ~0.45 cm³ (Capan-2 controls) to ~0.1 cm³ (5E1-treated mice).

Histologic and immunohistologic analyses of tumor sections revealed that inhibiting SHH with 5E1 significantly decreased the degree of desmoplasia. Figure 1 shows staining of tumor sections with H&E and with the mesenchymal marker for differentiated fibroblasts, α-SMA. The images in Fig. 1A are representative of the desmoplastic reaction in tumors derived from the Capan-2 cell line with and without inhibition of SHH by treatment with antibody 5E1. Tumors in animals treated with the control antibody, 4E11, showed dense desmoplastic reactions. Tumors derived from mice treated with the 5E1 showed minimal evidence of desmoplasia. We confirmed that areas of desmoplasia contained myofibroblasts by staining the tumor sections with SMA (Fig. 1B). Morphometric analysis revealed a significant decrease in the number of SMA⁺ cells between control tumors and tumors from mice treated with 5E1 (Fig. 1C).

**SHH expression promotes a desmoplastic reaction when overexpressed in transformed HPNE cells.** To further investigate the role of SHH in the production of pancreatic cancer desmoplasia, we overexpressed SHH in a transformed primary pancreatic epithelial cell line, T-HPNE. SHH overexpression...

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**Fig. 1.** Inhibition of SHH signaling decreases desmoplasia in Capan-2 orthotopic tumors. A, histologic analysis of tumors derived following orthotopic challenge with Capan-2 and treatment with a blocking antibody for SHH (5E1), an isotype control (4E11), or no treatment (Capan-2). H&E staining, at ×100 magnification, shows reactive stroma and desmoplasia in the Capan-2 and control 4E11-treated Capan-2 tumors (see black arrows). The 5E1-treated Capan-2 tumors showed little or no evidence of desmoplasia by histologic examination. B, immunohistochemistry with the mesenchymal marker SMA. Paraffin-embedded sections of tumors that arose from orthotopic implantation of Capan-2 cells and treatment with a blocking antibody for SHH (5E1), an isotype control (4E11), or no treatment (Capan-2). Brown coloration, positive staining for SMA. Counterstaining was done with hematoxylin (blue). C, morphometric quantification of the relative amount of SMA⁺ desmoplasia in the Capan-2 control, 4E11-treated, and 5E1-treated tumors. The 5E1 tumors showed a significant decrease in the amount of SMA⁺ desmoplasia. Relative SMA⁺ areas on the tumor sections were quantified by calculating the total cellular content of the section and expressing the results as a percentage: (SMA⁺ desmoplasia / all cell types in total area) × 100. Two representative results of 5E1-treated Capan-2 primary tumors showed some staining with SMA, but the areas of staining were individualized fibroblasts representing <20% of the section. Inhibition of SHH significantly decreased the amount of SMA⁺ desmoplasia in Capan-2 tumors (p = 5 per group).
in this cell line was confirmed by Western blot (Supplementary Fig. S1). T-HPNE and T-HPNE cells expressing SHH (T-HPNE.SHH) were implanted orthotopically into the pancreas of athymic nude mice. Both T-HPNE cells and T-HPNE.SHH formed poorly differentiated tumors at the orthotopic site. Expression of SHH in the T-HPNE cells resulted in an increase in mean tumor weight from \( \sim 1 \) g (T-HPNE) to \( \sim 3 \) g (T-HPNE.SHH). There was dense desmoplasia in tumors produced by the T-HPNE.SHH cell line, whereas desmoplasia was minimal in tumors derived from T-HPNE cells not expressing SHH, as evidenced by morphologic evaluation and immunohistochemical analysis of the orthotopic tumors derived from the T-HPNE.SHH cell line with SMA to confirm that cells in the desmoplastic reaction were myofibroblasts. Our analysis showed a significant increase in cells that stained positive for this mesenchymal marker within the tumor and at the periphery (Fig. 2A and B). Tumors derived from the T-HPNE cell line showed minimal reactivity with \( \alpha \)-SMA (Fig. 2A), and the reactivity was restricted to the area around pancreatic ducts, which have previously been shown to stain positive. Figure 2C shows morphometric analysis of tumors derived from the T-HPNE and T-HPNE.SHH cells and indicates a significant increase in desmoplasia in the tumors derived from the T-HPNE.SHH cells.

We stained adjacent sections with collagen I and fibronectin to determine if the myofibroblasts synthesized these two well-characterized components of desmoplasia. Figure 3A shows immunohistochemical staining of tumors derived from the T-HPNE.SHH cell line and confirms that the area containing myofibroblasts and desmoplasia also stained positive for fibronectin and collagen I. Moreover, there were few myofibroblasts in the tumors derived from the T-HPNE cell line (Fig. 3A), and immunohistochemical analysis of these sections showed an absence of collagen I and fibronectin.

In Fig. 3B, we confirmed the presence of collagen I and fibronectin by immunohistochemistry to stain for these
components in tumors derived from untreated mice challenged with the Capan-2 cell line. There is a decrease in both the intensity and quantity of both collagen I and fibronectin staining in the Capan-2 tumors derived from the mice treated with 5E1.

Myofibroblasts express Gli-1. We sought to determine if SHH was stimulating myofibroblasts by a paracrine mechanism. Figure 4A shows immunohistochemical analysis of human pancreatic cancer tissue obtained from autopsy, showing that...
tumor cells stain positive for SHH, whereas the surrounding fibroblasts and areas of desmoplasia were positive for Gli-1, whose expression is known to be induced following stimulation with SHH. These data support the hypothesis that fibroblasts that make up the desmoplastic reaction in human pancreatic cancer show evidence of paracrine signaling in response to SHH produced by human pancreatic cancer cells.

We also analyzed adjacent sections of T-HPNE-SHH tumors with antibodies to SMA and Gli-1, which showed evidence that SMA\(^+\) myofibroblasts expressed Gli-1, supporting the hypothesis that these cells were responding to SHH produced by the T-HPNE-SHH cells in a paracrine manner (Fig. 4B).

**Myofibroblasts and SMA\(^+\) cells are of murine origin.** We used a human-specific antibody to a ribonuclear protein and an antibody that binds to both murine and human SMA to determine if the SMA\(^+\) cells were of human or murine origin. Figure 5A presents confocal analysis of expression of these markers in orthotopic tumors derived from human T-HPNE, T-HPNE-SHH, Capan-2, and 5E1-treated Capan-2 tumors. The majority of these cells were clearly of murine origin and showed myofibroblastic morphology.

**SHH promotes the differentiation and proliferation of pancreatic stellate cells.** Confocal analysis of the 5E1-treated Capan-2 tumors (Fig. 5A) revealed single SMA\(^+\) cells embedded in the human tumors, raising the possibility that these were stellate cells that were progenitors to the myofibroblasts observed in Capan-2 tumors when SHH signaling was not inhibited (Fig. 5A). Capan-2 tumors were stained for cyclin D1, which was detected in the myofibroblasts, supporting the hypothesis that these represented stellate cells within the tumor that were proliferating in response to SHH stimulation (Fig. 5B).

We isolated pancreatic stellate cells from the human pancreas using previously described techniques (7, 10) and confirmed their lineage by PCR to identify markers of both epithelial and mesenchymal lineage. Consistent with the anticipated phenotype for pancreatic stellate cells, the isolated cells expressed higher levels of SMA and vimentin and displayed a spindleshape morphology (Supplementary Fig. S2). We stimulated these cells with rhSHH at 1 and 10 \(\mu\)g/mL for 24 hours and observed an increase in the mesenchymal markers SMA, vimentin, and desmin and a decrease in the epithelial marker cytokeratin 19 (Fig. 5C). These data are the first to suggest that SHH induces the differentiation of pancreatic stellate cells into myofibroblasts.

**SHH in vitro promotes the invasion of human pancreatic myofibroblasts.** We analyzed the ability of human myofibroblasts from the pancreas to respond to SHH in Matrigel invasion assays to determine if SHH contributes to increased desmoplasia by enhancing the motility and invasive capacity of

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**Fig. 4.** SHH paracrine signaling between tumor and stroma. A, representative immunohistochemical analysis of primary pancreatic ductal adenocarcinoma tissue sections from patients with pancreatic cancer. Brown color, positive staining for Gli-1 or SHH. We observed positive staining for SHH in the tumor cells, whereas there was minimal staining in the tumor cells for Gli-1 (black arrows, positive staining; white arrows, negative staining). We observed minimal staining for Gli-1 in the tumor cells and observed reactivity with the Gli-1 antibody in areas of desmoplasia. B, immunohistochemical analysis of T-HPNE-SHH tumors, showing both SMA and Gli-1 staining; \(\times 200\) magnification. The brown staining in adjacent sections indicates that the SMA\(^+\) myofibroblasts infiltrating the T-HPNE-SHH tumor section are expressing Gli-1 (\(n = 3\)).
these cells. Human pancreatic myofibroblasts, which express the markers vimentin and SMA and lack expression of cytokeratin 19 (Supplementary Fig. S3A), were isolated and shown to express Gli-1 when stimulated with SHH (Supplementary Fig. S3B). Figure 6A shows the results of a Matrigel invasion assay in which increasing numbers of T-HPNE and T-HPNE.SHH cells were seeded in the lower chamber and myofibroblasts were seeded in the upper chamber. For cultures containing 250,000 and 500,000 T-HPNE cells expressing SHH, there was a significant increase in the invasive capacity of myofibroblasts as compared with T-HPNE controls. We repeated this experiment with recombinant human SHH at either 1 or 10 μg/mL. SHH stimulation increased the expression of desmin, vimentin, and SMA and decreased the expression of cytokeratin 19.

We also conducted an in vitro wound healing assay to determine if SHH increased the ability of the myofibroblasts in a monolayer to migrate into a wound. Figure 6C and D shows the visual and quantitative results from this experiment. After 48 hours, SHH significantly increased the ability of the myofibroblasts to recovery from the wound (90% recovery with SHH versus 45% recovery in the control cells and cells with SHH and the 5E1 SHH inhibitor). These experiments support the hypothesis that SHH increases the migration and invasion of human pancreatic myofibroblasts in vitro.

**Discussion**

Hedgehog signaling was first identified and characterized in *Drosophila* and has been shown to play a role in organogenesis and differentiation. During vertebrate patterning, hedgehog...
signaling induces differentiation of the skeleton, central nervous system, early gut endoderm, limb axes and ventral somite buds (13, 14). Interestingly, SHH has not been shown to contribute to normal differentiation of the pancreas, and ectopic expression in the epithelia of the embryonic pancreatic mesoendoderm leads to malformation of the pancreatic mesoderm and an increase in the number of mesenchymal cells in the developing pancreatic compartment (4).

There are three known human hedgehog family members: Sonic (SHH), Indian (Ihh), and Desert (Dhh). After translation of the 45-kDa protein, the hedgehog proteins undergo an autocatalytic cleavage facilitated by a cholesterol modification, an event that yields a 19-kDa NH₂-terminal protein that is secreted and a 25-kDa COOH-terminal protein that remains associated with the cell (15, 16). The secreted ligand binds to a 12-pass transmembrane protein, Patched (Ptc), which relieves the inhibitory effect of Patched on another transmembrane protein, Smoothened (SMO; refs. 15–17), which in turn enables hedgehog signaling. The complex signaling cascade downstream of SMO activation is not well characterized, yet ultimately SHH transduces a signal that induces nuclear translocation of Gli, a target transcription factor, and the transcriptional activation of hedgehog target genes, including Ptc, Gli, and hedgehog interaction protein, Hhip (18, 19).

Several reports have implicated the misregulation of the hedgehog signaling pathway in the initiation and progression of pancreatic cancer (5, 20). Mutations in this pathway are associated with cancer initiation and progression, as mutations in the tumor suppressor Patched are characterized in basal cell carcinoma, medulloblastoma, and rhabdomyosarcoma, whereas oncogenic mutations in the downstream transcription factor Gli have been identified in glioblastoma and basal cell carcinoma (21, 22). Pancreatic cancer presents a different oncogenic insult within the hedgehog pathway, as SHH is upregulated in the tumor cells but absent in the normal pancreas.

We observed that expression of SHH was correlated with desmoplasia in two cell lines grown orthotopically in nude mice. Desmoplasia, a common and well-noted histologic feature of pancreatic cancer, has been proposed to contribute to progression of the disease (23); however, the molecular

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Fig. 6. SHH increases the in vitro invasive capacity of primary cells from the human pancreas. A, invasion assay in Matrigel invasion chambers. T-HPNE cells or T-HPNE:SHH cells were seeded in the lower chambers at densities of 50,000, 250,000, and 500,000 per well. With 250,000 and 500,000 cells, SHH secreted from the T-HPNE:SHH cell line significantly increased the invasive potential of an immortalized human pancreatic myofibroblast cell line (*, P < 0.05). Columns, mean number of invasive cells for each cell line (n = 3); bars, SE. B, invasion assay in Matrigel invasion chambers. Recombinant SHH in the lower chamber of the invasion assay significantly increased the invasive potential of an immortalized human pancreatic myofibroblast cell line (**, P < 0.01). Columns, mean number of invasive cells for each cell line (n = 3); bars, SE. C and D, in vitro wound healing assay. C, pictures of initial time point controls and 48-h time points showing the effects of SHH stimulation on pancreatic myofibroblasts. D, quantitative analysis of the in vitro wound healing assay (percent closure). SHH-stimulated immortalized human pancreatic myofibroblasts showed a significant increase in their ability to recover from the wound (*, P = 0.05). The addition of the 5E1 blocking antibody significantly inhibited the SHH-mediated wound healing ability back to control levels (**, P = 0.05). Values are shown for each cell line; bars, SD.
processes that cause the desmoplastic reaction are not well characterized. Previous experiments in prostate cancer have determined that SHH contributes to desmoplasia and tumor growth (24); thus, we propose that SHH has a similar mechanistic role in pancreatic cancer. We explored the requirement for SHH in the induction of desmoplasia by administering a SHH-neutralizing antibody, 5E1, to mice with Capan-2 cells injected orthotopically into their pancreas. Capan-2 tumors express SHH and produce a desmoplastic reaction when grown orthotopically in mice. Capan-2 tumors treated with the SHH-inhibiting antibody were smaller and did not elicit a desmoplastic response when compared with the control tumors derived from the Capan-2 cell line that received no treatment or an isotype control antibody. Similarly, SHH expression in T-HPNE cells (T-HPNE.SHH) produced larger tumors that showed significant differences in cellular morphology. Within the tumor sections of the T-HPNE.SHH cell line, we noted the presence of infiltrating fibroblasts as long, extended cells with elongated nuclei. This fibrotic infiltration was accompanied by expression of collagen I and fibronectin, and represented further evidence of a desmoplastic reaction within tumors from the SHH overexpressing transformed cell line (25, 26). This reaction was not observed in the tumor sections from the T-HPNE cell line.

We furthered evaluated the effects of SHH signaling in vitro on primary pancreatic myofibroblasts and found that SHH acted as a chemoattractant. This result complements previous studies, which showed that cancer cells recruit fibroblasts, and provides the first reported evidence that SHH, secreted from transformed pancreatic epithelia, contributes to the recruitment and organization of fibroblasts to enhance tumor progression (27). We also show that SHH can induce the differentiation and proliferation of human pancreatic stellate cells, which can also be a mechanism whereby SHH induces desmoplasia.

In summary, our data are significant in that they identify SHH as a regulator of one critical pathologic outcome of pancreatic ductal adenocarcinoma: the production of desmoplasia, or the infiltration of fibrous tissue into neoplasms, which results from the proliferation of fibroblasts and the increase in fibrosis within the stroma (28–30). Desmoplasia is a common histologic feature of many adenocarcinomas (31, 32). The stroma, or the complex microenvironment surrounding tumor cells, includes inflammatory cells, fibroblasts, extracellular matrix, and small blood and lymphatic vessels (33, 34). Previous experiments have shown that TGFβ is a mediator of fibrosis (12) in pancreatic cancer. In data not shown, we conducted a microarray on T-HPNE versus T-HPNE.SHH cells and determined that the expression of SHH did not up-regulate the expression of TGFβ. Thus, it is possible that SHH directly influences the desmoplastc reaction, but it remains possible that SHH acts by inducing signals that interact with TGFβ signaling pathways (35). We have also shown that SHH can act as a chemoattractant for myofibroblasts in invasion assays and in wound healing assays, where SHH increased the ability of pancreatic myofibroblasts to recover from a wound. These data suggest that SHH is a critical player in the tumor microenvironment. Further elucidation of its functions will lead to a better understanding of the pathogenesis of this lethal disease and the potential for therapeutic targeting of SHH to disrupt the microenvironment in pancreatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Sonic Hedgehog Promotes Desmoplasia in Pancreatic Cancer

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