Overexpression of Smoothened Activates the Sonic Hedgehog Signaling Pathway in Pancreatic Cancer–Associated Fibroblasts

Kimberly Walter1, Noriyuki Omura1, Seung-Mo Hong1, Margaret Griffith1, Audrey Vincent1, Michael Borges1, and Michael Goggins1,2,3

Abstract

Purpose: Accumulating evidence suggests that cancer-associated stromal fibroblasts (CAF) contribute to tumor growth by actively communicating with cancer cells. Our aim is to identify signaling pathways involved in tumor-stromal cell interactions in human pancreatic cancer.

Experimental Design: We established primary fibroblast cultures from human pancreatic adenocarcinomas and nonneoplastic pancreas tissues. To identify differentially expressed genes in CAFs, we did gene expression profiling of human pancreatic CAFs and nonneoplastic pancreatic fibroblasts.

Results: The Hedgehog receptor Smoothened (SMO) was upregulated in CAFs relative to control fibroblasts. CAFs expressing SMO could transduce the Sonic hedgehog signal to activate Gli1 expression, and small interfering RNA knockdown of SMO blocked the induction of Gli1 in these cells. Stromal fibroblasts of human primary pancreatic adenocarcinomas overexpressed Smo compared with normal pancreatic fibroblasts.

Conclusions: These findings implicate overexpression of Smo as a mechanism for the activation of Hedgehog signaling in human pancreatic CAFs and suggest that stromal cells may be a therapeutic target for Smo antagonists in pancreatic cancer. Clin Cancer Res; 16(6); 1781–9. ©2010 AACR.

Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States (1). It is one of the most highly invasive of the solid cancers and is characterized by an extensive desmoplastic stromal response (2). Mounting evidence suggests that cancer-associated fibroblasts (CAF), the predominant stromal cell type, actively communicate with and stimulate tumor cells, thereby contributing to tumor development and progression. Recent studies in multiple pancreatic cancer model systems have implicated the Hedgehog signaling pathway in these tumor-stromal interactions (3, 4).

The Hedgehog signaling pathway, a crucial regulator of proliferation and differentiation during embryonic development, has been reported to be aberrantly activated in many solid tumors, including basal cell carcinoma (5–7) and medulloblastoma (8), and, more recently, in several gastrointestinal cancers, including pancreatic cancer (9–12). Hedgehog proteins are secreted signaling molecules that can signal responsive cells at a significant distance from the producing cells. Three mammalian Hedgehog ligands have been described: Sonic hedgehog (Shh), Indian hedgehog, and Desert hedgehog. These ligands initiate Hedgehog signaling by binding to the Patched (Pch) 12-transmembrane domain receptor. Pch then activates Smoothened (Smo), a seven-transmembrane spanning protein and the central transducer of the Hedgehog signal. Activated Smo induces nuclear localization of the Gli family of transcription factors, resulting in transcription of Hedgehog-specific target genes, including Gli1 and Pch.

Constitutive activation of the pathway results in cell proliferation and tumor formation and commonly occurs as a result of activating mutations in SMO (13, 14) or inactivating mutations in the tumor suppressor gene Pch (5, 15). Mutations of Pch or SMO have not been described in pancreatic cancer (16), but overexpression of the Shh ligand has been reported to occur in 70% of primary pancreatic adenocarcinomas (12) and has been implicated in the development and progression of pancreatic tumors. Forced overexpression of Shh during mouse development results in formation of lesions resembling pancreatic cancer precursor pancreatic intraepithelial neoplastic lesions (12, 17). Cell lines established from primary and metastatic pancreatic cancers retain the expression of several components of the Hedgehog signaling pathway (3, 12).
Translational Relevance

This article describes the identification of differentially expressed genes in human pancreatic cancer–associated fibroblasts compared with pancreatic fibroblasts from controls and identifies overexpression of the Hedgehog pathway member, Smoothened (Smo). We further describe evidence of activation of the Hedgehog pathway in pancreatic cancer–associated fibroblasts that is dependent on Smo expression. These findings suggest that Smo overexpression is the mechanism of activation of the Hedgehog pathway in human pancreatic cancer fibroblasts and support the rationale for using Smo antagonists in the treatment of patients with pancreatic cancer.

The plant-derived teratogen cyclopamine, which inhibits Smo activity, suppresses growth of these cell lines both in vitro and in vivo (12). Furthermore, cyclopamine therapy inhibits development of tumor metastases in xenografted mice (10, 18) and prolongs survival in a mouse model of pancreatic cancer (19). These data support a functionally important role for Hedgehog signaling in pancreatic ductal tumorigenesis.

Previously, a cell-autonomous role for Hedgehog signaling has been described in tumor types driven by mutations in Hedgehog pathway components, such as medulloblastoma and basal cell carcinoma (20). However, an alternative mechanism, in which tumor cell–derived Hedgehog ligands stimulate neighboring stromal cells, has recently been described in mouse models of pancreatic cancer. To identify signaling pathways involved in tumor-stromal cell interactions in human pancreatic cancer, we have now established primary CAF cultures from human pancreatic adenocarcinomas and nonneoplastic pancreas tissues. By doing global gene expression analysis of pancreatic CAFs versus fibroblasts from nonneoplastic pancreas using Affymetrix exon microarrays, we identified the Hedgehog receptor SMO as overexpressed in human pancreatic CAFs. Overexpression of Smo protein was confirmed by immunohistochemical staining in stromal fibroblasts of primary human pancreatic adenocarcinomas. We also present evidence of Hedgehog pathway activity in stromal cells derived from primary pancreatic adenocarcinomas. Our results implicate overexpression of Smo as a mechanism for Hedgehog signaling in the stromal cells of pancreatic ductal adenocarcinomas.

Materials and Methods

Culture of cell lines and establishment of fibroblast cultures. Primary cultures of stromal fibroblasts, designated CAFs CAF11, CAF12, CAF13, CAF15, CAF16, CAF18, CAF19, CAF20, CAF21, CAF22, CAF25, CAF26, CAF27, CAF37, CAF38, CAF39, and CAF40, were established as previously described (21) from surgically resected pancreatic cancer tissue from 17 patients (8 males and 9 females with a mean ± SD age of 64 ± 12 y) with clinically sporadic pancreatic ductal adenocarcinoma. The cancers were all moderate to poorly differentiated with a mean tumor size of 3.4 cm. Cells were grown at 37°C in a humidified atmosphere containing 5% CO2. All CAFs were used at early passage numbers (passages 3-6). Nine CAFs were used for microarray analysis. Because these CAFs senesced after several passages, additional primary CAFs were generated for use in subsequent experiments. The packaging cell line Phoenix A (kindly provided by Dr. C. Dang and Dr. T.C. Wu, Johns Hopkins University, Baltimore, MD) was maintained in DMEM containing 10% fetal bovine serum (FBS) and 2% penicillin-streptomycin (Invitrogen). Human pancreatic ductal epithelial cells, generously provided by Dr. Ming-Sound Tsao (University of Toronto, Toronto, Ontario, Canada), were also maintained in DMEM containing 10% FBS and 2% penicillin-streptomycin and used as a control line for immunohistochemical staining (22). All samples were collected with approval from the Johns Hopkins Committee for Clinical Investigation.

Immortalization of pancreatic fibroblasts. The human pancreatic Nestin-expressing cells (HPNE) generously provided by Dr. Michel M. Ouellette (University of Nebraska Medical Center, Omaha, NE) were used as a control cell line. These cells were derived from normal pancreatic tissue and immortalized using hTERT (23).

Two immortalized control fibroblast cultures (SC2 and SC3) were also established from nonmalignant pancreatic tissues. SC2 was derived from an intraductal papillary mucinous neoplasm (IPMN) of the pancreas, and SC3 was derived from resected chronic pancreatitis tissue. Immortalized fibroblasts (SC2 and SC3) were generated by transduction of primary normal fibroblast cultures with an hTERT-expressing retrovirus. The amphotropic packaging cell line Phoenix A was transiently transfected with 5 μg of the pBABE-puro-hTERT (Addgene plasmid 1771) retroviral vector generated according to Counter et al. (24). After 24-h transfection in Lipofectamine 2000 and OptiMEM (Invitrogen), the cells were incubated for 48 h at 37°C in complete medium (DMEM containing 10% FBS). The viral supernatants were then filtered through a 0.45-μm filter and used to infect target cells in the presence of 8 μg/ml polybrene (Sigma). A second round of viral infection was carried out 72 h after the first infection. The infected cells were subsequently selected by growing them in the presence of 1 μg/ml puromycin (Sigma).

Sample preparation and affymetrix exon array analysis. Total RNA was isolated from CAF and control fibroblast cultures using a Qiagen kit or an Ambion mirVana miRNA kit according to the manufacturer's instructions. The Affymetrix GeneChip Human Exon 1.0 ST Array platform was used to analyze gene expression patterns in CAFs and control fibroblasts on a whole-genome scale. Using the GeneChip Whole Transcript Sense Target Labeling Assay (Affymetrix), 1 μg of total RNA from each CAF culture or control fibroblast culture was processed, labeled, and
The expression of the housekeeping gene transcript levels in normal fibroblast and CAF samples. The expression level of the housekeeping gene 18s rRNA (Applied Biosystems) was used for normalization. Results

**Quantitative reverse transcription-PCR.** Total RNA (1 µg) was reverse transcribed using avian myeloblastosis virus reverse transcriptase and random primers (Promega) according to the manufacturer's instructions. The resulting cDNA was amplified on the ABI 7300 Real-Time PCR thermocycler (Applied Biosystems) using Taqman Universal PCR Master Mix and recommended PCR conditions (Applied Biosystems) to quantitatively assess gene transcript levels in normal fibroblast and CAF samples. The expression level of the housekeeping gene 18s rRNA (Applied Biosystems) was used for normalization. Results are expressed as normalized values relative to the indicated cell line (2^−ΔΔCt). All PCRs were done in triplicate. The following primer sets were used for Taqman assays (Applied Biosystems): Taqman Gene Expression Assays Hs00170665_m1 (hSmo), Hs00179843_m1 (hShh), Hs00171790_m1 (hGli1), Hs00257977_m1 (hGli2), and 18s rRNA VIC-MGB probe dye. 

Small interfering RNA knockdown of Smo in CAF cultures. CAFs were plated in DMEM containing 10% FBS and 2% penicillin-streptomycin at a density of 5 × 10^4 cells per well in a 24-well plate and transfected with 2 µmol/L Smo or nontargeting control pool small interfering RNA (siRNA; Dharmacon) using the DharmaFECT4 transfection reagent (Dharmacon) in Opti-MEM. RNA was extracted 72 h after transfection using Trizol (Invitrogen). RNA was reverse transcribed to cDNA using random primers and avian myeloblastosis virus reverse transcriptase.

ShhN ligand stimulation of CAF cultures. CAF or control fibroblast cells were plated in complete medium (DMEM + 10% FBS) at a density of 1 × 10^4 in a 24-well plate and grown to confluence. The cells were incubated for 24 h in low-serum medium (DMEM + 0.5% FBS) containing 0, 0.5, 1.0, or 2.5 µg/mL of recombinant human Shh (ShhN) ligand (R&D Systems, Inc.). Recombinant ShhN ligand was added to the siRNA-transfected cells at 48 h after transfection, and the cells were incubated for 24 h in low-serum medium. RNA was extracted using Trizol and assayed for the expression of Shh target genes.

Pancreatic adenocarcinoma tissue microarrays and SMO immunohistochemistry. The expression of the SMO protein was examined by immunohistochemical labeling of formalin-fixed, paraffin-embedded whole tissue sections or tissue microarrays using a DAKO Autostainer. Eight tissue microarrays containing a total of 156 different surgically resected pancreatic ductal adenocarcinomas, IPMNs, and chronic pancreatitis tissues were constructed as previously described (25). All specimens were collected and analyzed with the approval of the Johns Hopkins Committee for Clinical Investigation. Tissue sections were deparaffinized in xylene, hydrated in graded ethanol concentrations, and boiled for 20 min in epitope retrieval buffer. Immunostaining was then done on the DAKO Autostainer using a rabbit polyclonal anti-human SMO antibody (Abcam, Inc.) at a 1:800 dilution, a mouse monoclonal anti-vimentin antibody (clone V9; DAKO) at a 1:100 dilution, a mouse monoclonal anti-cytokeratin 19 antibody (Santa Cruz Biotechnology) at a 1:100 dilution, or a mouse monoclonal anti-α-smooth muscle actin (SMA) antibody (clone IA4; DAKO) at a 1:100 dilution for an incubation time of 60 min. Labeling was done according to the manufacturer's protocol using the Envision Plus Detection kit (DAKO). Nuclei were counterstained with hematoxylin. For immunocytochemical experiments, cells were cultured on chamber slides (BD Falcon) and grown until subconfluent. Cells were then fixed either in 10% formalin (for Oil Red O staining) or in 70% methanol (for immunostaining) and subjected to Oil Red O staining for 15 min or to immunostaining on the DAKO Autostainer. To quantify SMO expression in stromal fibroblasts, three consecutive sections from one tissue microarray were stained with antibodies against vimentin and α-SMA to identify fibroblasts and activated fibroblasts, respectively, and against SMO. Sixty-one cores from pancreatic cancer tissue and 24 cores from normal pancreatic tissue were scored by two independent researchers (S-M.H. and K.W.) for expression of SMO in stromal fibroblasts. An average of 40 fibroblasts from each individual core was scored, and the percentage of SMO-positive fibroblasts was calculated. Cells with a weak positive signal were considered positive for SMO expression, and cores containing any areas of focal expression were considered positive for SMO expression.

Statistical analysis. Descriptive statistical values and plots were generated using the Microsoft Excel software package, and the SPSS Mann-Whitney (nonparametric test) and Pearson χ^2 tests were done using SPSS 17.0 software statistical program or the Partek Genomics Suite version 6.3 beta. For the quantitative reverse transcription-PCR (qRT-PCR) experiments, statistical analysis was done with Microsoft Excel software using a paired Student's t test (two-tailed). Differences were considered significant at P < 0.05, and values reported are means ± SD. For microarray experiments, gene expression analysis was done using Partek Genomics Suite version 6.3 beta. The Robust Multichip Average method was used to normalize the raw intensity measurements of all probe sets. Gene expression values were then obtained using the one-step Tukey’s
biweight method. Two-way ANOVA was done to identify significant expression changes between CAFs and control fibroblasts using a fold change criterion of 2.8-fold and a P value of <0.05. Gene ontology analysis was done using the Biological Interpretation feature of the Partek software (version 6.5).

Results

Characterization of pancreatic fibroblasts. The pancreatic fibroblasts isolated from benign and malignant primary pancreatic resection specimens expressed the mesenchymal-marker vimentin by immunocytochemistry (Supplementary Fig. S1). Both the pancreatic CAFs and the fibroblast lines SC2 and SC3 from nonneoplastic pancreas appear as myofibroblasts with both a spindle-shaped and stellate morphology (Supplementary Fig. S2). Pancreatic CAFs contain lipid droplets and stain positive for vimentin and α-SMA, a marker for activated fibroblasts (Supplementary Fig. S2).

SMO is upregulated in pancreatic CAFs. To identify genes that are differentially expressed in pancreatic CAFs in pancreatic CAFs relative to control fibroblasts, we compared the global gene expression profiles of nine CAFs (CAF11, CAF12, CAF13, CAF15, CAF16, CAF18, CAF19, CAF21, and CAF22) to control fibroblast lines derived from non-neoplastic pancreas (HPNE and SC3) and from an IPMN (SC2). The nonneoplastic pancreatic cell line HPNE was used as a control fibroblast line as it has fibroblast morphology, expresses vimentin, and does not express the ductal marker vimentin by immunocytochemistry (Supplementary Table S1). A gene ontology analysis of the genes upregulated in pancreatic CAFs revealed that these fibroblasts and not pancreatic ductal cells by principal component analysis (data not shown).

Gene expression profiles were obtained using the Affymetrix Exon Array ST 1.0. We first examined the top 200 candidate genes that were expressed at significantly higher levels (∼2.8-fold or greater; P < 0.05, ANOVA) in CAFs compared with control fibroblasts. This criterion identified several genes that have previously been described in tumor-stromal interactions such as ADAM12, IL1α, and POSTN (28). ADAM12 and IL1α were upregulated in pancreatic CAFs (Fig. 1B). We confirmed the overexpression of IL1α mRNA in pancreatic CAFs by using qRT-PCR. IL1α mRNA levels were low or undetectable in the control fibroblasts HPNE and SC2, and modest expression was detected in the control fibroblast line SC3. In contrast, higher levels of SMO mRNA were detected in all 15 CAFs tested (Fig. 1B). Relative levels of SMO mRNA correlated closely with the results obtained by exon array analysis (Fig. 1A).

Pancreatic CAFs also expressed the Hedgehog pathway components Ptc1, Gli1, and Gli2 mRNA (data not shown), but did not express Shh mRNA, consistent with previous studies of human (3, 4) and mouse fibroblasts (3, 4, 31).

Shh ligand induces expression of Gli1 mRNA in pancreatic CAFs. To determine if pancreatic CAFs are responsive to Shh signaling, we treated pancreatic CAFs with recombinant ShhN ligand. Because expression of the transcription factor GLI1 is a reliable marker of Hedgehog pathway activity, we measured Gli1 mRNA expression by qRT-PCR in response to ShhN treatment (Fig. 1B). Because all the CAFs tested had overexpression of ShhN, we selected three CAFs growing in culture (CAF25, CAF27, and CAF38) for ShhN ligand treatment. Exogenous ShhN ligand treatment for 24 hours resulted in a ∼2-fold induction of Gli1 mRNA (Fig. 2).

In contrast, ShhN was unable to induce Gli1 expression in HPNE or SC2 cells that express low or undetectable levels of SMO.

siRNA knockdown of SMO expression blocks GLI1 induction in pancreatic CAFs. To further investigate the role of SMO expression in transducing the Shh signal, we transiently knocked down SMO expression in another overexpressing CAF, CAF26, and then stimulated them with ShhN ligand. Using siRNA, we were able to knock down SMO with 90% efficiency (Fig. 3A), which resulted in a 70% (P = 0.007) reduction in Gli1 mRNA expression in CAF26 (Fig. 3B). We also found that reducing SMO expression blocked the induction of Gli1 mRNA expression in response to ShhN treatment. ShhN treatment induced Gli1 mRNA expression in a dose-dependent manner in CAF27 cells that were either mock transfected or transfected with nontargeting siRNA but could not induce Gli1 mRNA expression in cells with siRNA-mediated SMO knockdown (Fig. 3C).

Smo protein is expressed in stromal fibroblasts in primary human pancreatic tissues. Having verified that cultured pancreatic CAFs overexpress SMO mRNA, we did immunohistochemical labeling of human primary pancreatic ductal adenocarcinomas to determine whether overexpression of SMO protein occurs in stromal fibroblasts in vivo (Fig. 4). We used Purkinje neurons in cerebellar sections of brain tissue as a positive control for Smo staining (Fig. 4A). Renal epithelial cells of the distal convoluted tubule also stained Smo (data not shown). No significant staining was observed with the secondary antibody alone (Fig. 4C).

We analyzed Smo expression in normal fibroblasts and CAFs from whole tissue sections of the pancreas. Immunohistochemical staining of consecutive tissue sections confirmed that the vimentin-positive fibroblasts surrounding the normal pancreatic duct lacked expression of Smo (Supplementary Fig. S4), whereas the activated CAFs expressing α-SMA stained positive for Smo (Supplementary Fig. S5). We then analyzed 53 primary pancreatic cancers,
35 IPMN tumors, and 52 chronic pancreatitis tissue samples on tissue microarrays. Stromal fibroblasts surrounding the normal pancreatic duct were negative for SMO expression (Fig. 4A), and stromal fibroblasts in chronic pancreatitis tissue were weakly positive for SMO expression in 38 of 52 (73.0%) of cases (data not shown). This finding is consistent with our observation that SC3, a fibroblast cell line established from chronic pancreatitis tissue, weakly expresses Smo mRNA. It is possible that similar mechanisms are responsible for the fibroblast overexpression of Smo in chronic pancreatitis and in pancreatic cancer. We also frequently detected Smo expression in fibroblasts associated with IPMN tumors (28 of 35 or 80.0% of cases), although their expression...
levels were generally weaker than those in CAFs (data not shown). We observed a heterogeneous pattern of Smo expression in the stromal fibroblasts of pancreatic cancers. Whereas fibroblasts surrounding the normal pancreatic duct lacked Smo expression (Fig. 4B), CAFs stained positive for Smo, as indicated in Fig. 4D, a representative pancreatic adenocarcinoma strongly expressing Smo in the stromal fibroblasts. In the majority of pancreatic cancer cases, we observed a variable pattern of staining throughout the tumor, with areas of minimal staining interspersed with focal areas of intense staining of Smo in the fibroblasts. Fifty-one of 53 (96.2%) of pancreatic cancers evaluated contained areas with stromal expression of Smo. We observed Smo expression in pancreatic cancer cells (Fig. 4D; Supplementary Fig. S5B and D) and weak expression in normal ductal cells, consistent with previous reports that Smo is only occasionally detected in normal duct epithelium but is frequently detected in neoplastic epithelium (12, 32). We also observed weak staining of Smo in acinar cells, consistent with previous reports (12). We further quantified Smo expression in stromal fibroblasts by counting a percentage of Smo-positive fibroblasts in cores from both normal pancreas and pancreatic cancer tissues. An average of 75.5 ± 16.6% of CAFs and 10.5 ± 13.9% of fibroblasts from normal pancreatic tissue were positive for Smo protein expression ($P = 9.9 \times 10^{-19}$).

**Discussion**

Using Affymetrix exon arrays, we find that human pancreatic CAFs overexpress the Shh receptor Smo. This overexpression was confirmed in vivo because stromal fibroblasts in human primary pancreatic adenocarcinomas overexpress Smo protein relative to fibroblasts in normal
pancreas. The Hedgehog pathway has been identified as activated in CAFs in mouse models of pancreatic cancer. Our results implicate overexpression of Smo as a mechanism responsible for the activation of the Hedgehog pathway in human pancreatic CAFs. Although it remains unclear how paracrine Hedgehog signaling of fibroblasts contributes to tumor growth, recent work suggests that targeting Smo in the tumor stroma may be an effective strategy in treating pancreatic cancer. Previous work identified Shh as a mediator of the desmoplastic response in pancreatic cancer and suggested that the stroma may serve as a barrier to delivery of therapeutic compounds (33). Indeed, Olive et al. (34) recently reported that mice treated with the cyclopamine derivative and Smo inhibitor IPI-926 exhibited depletion of desmoplastic stroma and improved perfusion and delivery of chemotherapeutic drugs to pancreatic tumor cells, thereby increasing the survival time in these mice. Our immunohistochemical data indicate that these results are likely to be relevant to the treatment of human pancreatic cancer. We frequently observed Smo overexpression in the tumor stroma, suggesting that the stromal cells in human pancreatic cancers may be sensitive to Smo inhibition.

We showed that pancreatic CAFs can actively transduce the Hedgehog signal to induce GLI expression. CAFs expressing SMO respond to exogenous Hedgehog ligand, whereas control fibroblasts lacking SMO expression are unresponsive to Hedgehog ligand, and downregulation of SMO in CAFs inhibits transduction of the Hedgehog signal. Our work is consistent with recent studies in mouse models of pancreatic cancer showing a paracrine mechanism of Hedgehog signaling in cancer-associated stromal cells. First, expression of an oncogenic allele of Smo (SmoM2) in the mouse pancreas was unable to activate the Hedgehog pathway in ductal epithelial cells but resulted in Hedgehog signaling in adjacent stromal cells in several mouse models of pancreatic cancer (4). Second, coculture of Hedgehog-producing pancreatic cancer cell lines with 10T1/2 fibroblasts resulted in GLI reporter activity in the fibroblasts, showing the capacity of tumor cells to induce paracrine signaling (3). This mechanism was also observed in vivo in a xenograft model established from Hedgehog-expressing pancreatic cancer cell lines, in which Hedgehog pathway activation was detected in mouse stromal cells immediately adjacent to the xenografted tumors (3). Our findings that GLI expression can be induced in human pancreatic CAFs support these data and further show an intact canonical Hedgehog signaling pathway in human CAFs. Third, genetic deletion of Smo in mouse embryonic fibroblasts inhibited GlI1 induction.

Fig. 3. Effects of Smo siRNA and recombinant ShhN treatment on GlI1 mRNA expression in pancreatic CAFs. Smo (A) and GlI1 (B) levels were assessed by qRT-PCR in CAF26 cells transfected with 2 μmol/L of nontargeting or Smo siRNA for 72 h. C, CAP27 cells were transfected with 2 μmol/L of nontargeting or Smo siRNA and treated with the indicated concentrations of recombinant ShhN 48 h later. GlI1 mRNA levels were assessed at 72 h after transfection. Relative Smo or GlI1 mRNA levels after normalization to the corresponding 18S rRNA levels are shown. Columns, mean of three independent experiments; bars, SD.
in response to Hedgehog stimulation and resulted in decreased tumor growth of xenografts coinjected with these mouse embryonic fibroblasts (3). Similarly, we find that siRNA knockdown of SMO expression in CAFs results in decreased GLI1 expression, supporting a role for SMO overexpression in Hedgehog pathway activation. Taken together with these data, our work shows ligand-dependent Hedgehog pathway activation in the stromal microenvironment and supports a paracrine mechanism of Hedgehog signaling in human pancreatic cancer.

Not surprisingly, we observed increased GLI activity in response to ShhN stimulation in cells with higher levels of SMO expression. Our finding that ShhN is unable to induce GLI1 expression in normal control fibroblasts that do not express SMO is consistent with previous reports that cells lacking SMO expression lack the ability to receive the Hedgehog signal (35). Furthermore, siRNA knockdown of SMO blocked the ability of CAFs to induce GLI1 expression, indicating that Smo directly transduces the Hedgehog signal in these cells. Interestingly, we observed GLI1 expression in SC2 (IPMN-derived) fibroblasts lacking SMO expression (data not shown), consistent with recent reports of SMO-independent GLI1 transcription through noncanonical activation of the Hedgehog pathway (31, 36). It is also interesting that although GLI1 transcription increases on ShhN stimulation, CAFs overexpressing SMO also expressed detectable Gli1 levels in the absence of Hedgehog ligand (data not shown). This finding agrees with the observation that NIH-3T3 cells in which Smo is transiently overexpressed (37) as well as mouse pancreatic fibroblasts overexpressing SmoM2 (4) have increased Gli expression in the absence of Shh.

In summary, we find that human pancreatic CAFs overexpress the Hedgehog receptor SMO. Increased SMO expression denotes increased Hedgehog pathway activity in these cells, and we provide evidence of Hedgehog pathway activity in pancreatic cancer–associated stromal cells in vivo. These data contribute to a growing body of evidence that the Hedgehog pathway acts through a paracrine mechanism in human pancreatic cancer. SMO overexpression in the stromal compartment of human primary pancreatic adenocarcinomas suggests a tumor-stromal mechanism of Hedgehog pathway activation in vivo and may represent a therapeutic target in human pancreatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

National Cancer Institute grants CA62924 and CA120432 and Michael Rolfe Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 07/20/2009; revised 01/15/2010; accepted 01/16/2010; published OnlineFirst 03/09/2010.
References

Clinical Cancer Research

Overexpression of Smoothened Activates the Sonic Hedgehog Signaling Pathway in Pancreatic Cancer—Associated Fibroblasts

Kimberly Walter, Noriyuki Omura, Seung-Mo Hong, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-09-1913

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2010/03/16/1078-0432.CCR-09-1913.DC1

Cited articles
This article cites 36 articles, 14 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/16/6/1781.full#ref-list-1

Citing articles
This article has been cited by 16 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/16/6/1781.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.