Hedgehog signaling is restricted to the stromal compartment during pancreatic carcinogenesis

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The Hedgehog (Hh) pathway has been implicated in pancreatic cancer but its role remains controversial. To delineate the cell populations able to respond to Hh ligand stimulation, we expressed an oncogenic allele of *Smoothed* (*SmoM2*) to cell autonomously activate Hh signaling in the mouse pancreas. Surprisingly, we found that expression of *SmoM2* in epithelial cells was not able to activate the pathway and had no impact on pancreatic development or neoplasia. In contrast, activation of *Smo* in the mesenchyme led to Hh pathway activation, indicating that only the tumor stroma is competent to transduce the Hh signal. Using a *Ptc-LacZ* reporter mouse, we show that Hh signaling is active in stromal cells surrounding Hh-expressing tumor epithelium in various mouse pancreatic cancer models. Activation of the Hh pathway in the tumor stroma of human pancreatic and metastatic cancer specimens was confirmed by quantitative RT-PCR of microdissected tissue samples. These data support a paracrine model of Hh-mediated tumorigenesis, in which tumor cells secrete Hh ligand to induce tumor-promoting Hh target genes in adjacent stroma.

pancreatic cancer | paracrine | tumor stroma | *SmoM2* | *Kras*

Pancreatic ductal adenocarcinoma (PDA) is one of the most aggressive forms of cancer in the world, with a 5-year survival rate of less than 5%. Potential precursors of PDA include exocrine neoplastic changes, such as pancreatic intraepithelial neoplasms (PanINs), which demonstrate more severe epithelial atypia as they progress toward malignancy. Genetic analyses have linked mutations in human *KRAS* to PDA (1), and the functional role of oncogenic *KRAS* in both PDA initiation and progression were subsequently confirmed using genetically engineered animal models of pancreatic cancer (2–9). In addition to *KRAS*, a number of signaling pathways, including Wnt, TGF α , TGF β , Notch, EGF, and most recently, Hedgehog (Hh) have been implicated in PDA (10–15).

Paracrine Hh signaling plays a critical role during the development and maintenance of many epithelial appendages, including derivatives of the gut (16). This involves Hh ligand being secreted from the epithelium and binding to the Hh receptor Patched1 (*Ptch1*) in the adjacent mesenchymal receiving cells, which leads to relocalization of *Smoothed* (*Smo*) to primary cilia and activation of the Gli family of transcription factors (17). While there is little evidence for Hh pathway activity within the normal adult human exocrine pancreas (13), the expression of Hh ligands and essential components of the pathway, including *PTCH* and *SMO*, have been reported in up to 70% of human PDA specimens (13). In addition, several studies have proposed that Hh ligands produced by tumor cells activate Hh signaling and tumor cell growth in an autocrine/juxtacrine manner (13–14). Recently, using xenograft tumor models, our laboratory demonstrated a paracrine paradigm for Hh-mediated tumorigenesis, where Hh ligands produced by tumor cells act on the stromal compartment to support tumor growth indirectly (18). These data are consistent with the absence of mutations in canonical Hh pathway components that are commonly found in cell-autonomous, Hh-pathway-driven, tumor types, including medulloblastoma, basal cell carcinoma, and rhabdomyosarcoma (17).

In this study, we set out to further define a potential stromal versus epithelial role for Hh signaling in autochthonous pancreatic mouse and human cancers. Although several mouse models have

been established previously to investigate a causative role of Hh signaling in pancreatic tumorigenesis, none of these distinguished paracrine versus autocrine canonical Hh signaling in PDA. Among these models, ectopic expression of Sonic hedgehog (*Shh*) in the pancreas (*Pdx-1-Shh*) led to intestinal metaplasia with cellular atypia (13, 19) and a mouse with pan-epithelial expression of a dominant active *Gli2* allele (*CLEG2*) developed invasive, undifferentiated, pancreatic tumors (14). For the purposes of identifying the cell populations within the pancreas that are activated by Hh ligands, we chose to express in the pancreas an oncogenic allele of *Smo*, *SmoM2*, because this mutation activates the canonical Hh signaling pathway in a cell-autonomous manner.

Using this model in combination with a *Ptc-lacZ* reporter allele, we show here that the pancreatic epithelium is unable to transduce the canonical Hh signal. In contrast, we demonstrate that Hh signaling is restricted principally to smooth muscle actin (SMA)-expressing spindle cells adjacent to Hh-producing tumor epithelium. Quantitative RT-PCR on laser capture microdissected (LCM) tumor samples from both mice and humans confirm Hh signaling in the tumor stroma. Taken together, these results demonstrate that the pancreatic epithelium is not receptive of tumor cell-derived Hh ligands, but instead, Hh ligands promote PDA via a paracrine signaling mechanism received by tumor stromal cells.

Results

Epithelial Expression of *SmoM2* Is not Sufficient to Induce Neoplastic Transformation in Mouse Pancreatic Epithelium. The activating mutation W535L in human *SMO* (*SMOM2*) was initially identified in human basal cell carcinoma, and its ectopic expression causes constitutive, ligand-independent activation of the Hh pathway (20). To investigate whether Hh signaling in pancreatic epithelium is sufficient to induce pancreatic cancer, we used a previously described mouse model (21), *R26-SmoM2*, in which a cDNA fragment encoding the *SmoM2*-YFP fusion protein was targeted into the ubiquitously expressed *Rosa26* locus (*R26*) behind a stop cassette flanked by *Loxp* sites. YFP fused at the C terminus of *SmoM2* does not interfere with its activity in neural epithelium and provides a tool to trace the lineage of all Cre-expressing cells and their progeny (22).

We crossed *R26-SmoM2* with *PdxCre* transgenic mice (2) to activate Hh signaling within the pancreatic epithelium (Fig. 1A). *PdxCre;SmoM2* mice were born at Mendelian frequencies and showed no gross abnormalities up to 18 months of age. Microscopic examination of 12-week-old mice showed normal lobular pancreatic architecture with expression of both exocrine (cytokeratin 19 or CK19) and endocrine (insulin) markers (Fig. S1). Significantly, none of the *PdxCre;SmoM2* mice developed pancreatic neoplasms,

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Fig. 2. SmoM2 and Kras^{G12D} cooperate to induce pancreatic mesenchymal tumors in *PdxCre;Kras^{G12D};SmoM2* mice. (A) SMA expression by mesenchymal tumor in a *PdxCre;Kras^{G12D};SmoM2* mouse. (B) Vimentin expression by mesenchymal tumor in a *PdxCre;Kras^{G12D};SmoM2* mouse. (C) Muscular blood vessel in the pancreas of a control (*PdxCre;Kras^{G12D}*) mouse at 4 weeks. (D) Small, mesenchymal tumor arising from a muscular blood vessel in the pancreas of a 4-week-old *PdxCre;Kras^{G12D};SmoM2* mouse. Arrow highlights tumor extension into surrounding soft tissue. (E) Arteries in control *PdxCre;Kras^{G12D}* animal at 8 weeks. (F) Artery and mesenchymal tumor in 8-week-old *PdxCre;Kras^{G12D};SmoM2* mouse (MT* highlights tumor). (G) X-gal staining highlights the stochastic activity of *PdxCre* recombinase in pancreatic epithelium (duct, acini, and islet) when combined with the *R26-LSL-LacZ* reporter. (H) *PdxCre* activity is occasionally detected in adventitia surrounding blood vessels in *PdxCre;R26R* mice. (I) SmoM2-YFP

activation is not because of the low expression level of SmoM2 transgene, as we can readily detect accumulation of SmoM2-YFP fusion protein in both the exocrine (acini and ducts) and endocrine (islets) portions of the pancreas by immunofluorescence (see Fig. 1B). Interestingly, SmoM2 was also detected in primary cilia of both duct (see Fig. 1F) and islet cells.

In contrast, β -galactosidase expression was seen in mesenchymal cells, marked by vimentin staining, adjacent to pancreatic ducts in both control and *SmoM2* transgenic mice (Fig. 1D). Because these cells did not express SmoM2-YFP (see Fig. 1F), the β -galactosidase activity likely reflects the reception of Hh signals from ligands expressed by the ductal epithelium that are not detectable by immunohistochemistry (IHC) (13). This mesenchymal Hh signal surrounding the ducts was not affected by *SmoM2* oncogene expression in the pancreatic epithelium, as comparable levels of β -galactosidase staining were observed in both *PdxCre;Ptc-LacZ* and *PdxCre;SmoM2;Ptc-LacZ* mice.

Pancreatic Ductal Epithelial Cells Are Incompetent to Transduce Hh Signaling in Vitro. To further validate the inability of ductal epithelial cells to transduce the Hh signal, we treated a pancreatic ductal epithelial cell (PDEC) line, established from *PdxCre;Kras^{G12D}* mice, with recombinant Shh (rShh) or with a small molecule agonist of Smo (27). Similar to the observations in vivo, *Gli1* mRNA levels were not up-regulated in PDECs in response to ligand stimulation. In contrast, rShh or agonist treatment led to a 10- to 50-fold *Gli1* mRNA increase in primary pancreatic fibroblasts (Fig. 1G). This was not a result of the lack of native *Smo* expression in ductal cells because we detected similar levels of *Smo* mRNA in both ductal and fibroblast cells by RT-PCR (data not shown). Consistent with these results, electroporation of *SMOM2-GFP* in both fibroblasts and PDECs led to *Gli1* up-regulation only in fibroblasts (Fig. 1H). Therefore, failure to increase Hh signaling is most likely attributable to a block downstream of Smo in PDECs.

SmoM2 Does not Potentiate Kras^{G12D}-Driven PDA. While expression of SmoM2 alone in pancreatic epithelium was unable to activate the Hh pathway and did not initiate tumorigenesis, we asked whether SmoM2 could stimulate Hh signaling in pancreatic tumor epithelial cells and potentially accelerate the course of PDA tumor progression driven by oncogenic Kras^{G12D}. The expression of oncogenic Kras^{G12D} in murine pancreatic epithelial cells (*PdxCre;Kras^{G12D}*) drives the development of PanINs as early as 4 weeks of age, but most lesions remain low grade and progression to malignant PDA occurs slowly during the following year (2). *PdxCre;Kras^{G12D}*; *SmoM2* mice appeared normal at birth and did not display higher grade PanINs when compared with *PdxCre;Kras^{G12D}* littermates. However, *PdxCre;Kras^{G12D}*; *SmoM2* mice developed gastrointestinal obstructions and had to be killed by 24 weeks of age. These gastrointestinal obstructions were secondary to mesenchymal tumors, arising within both the pancreas and stomach (with 100% and 80% penetrance, respectively, $n = 30$), reflecting previously documented extra-pancreatic *PdxCre* transgene activity (2, 9). These mesenchymal tumors stained positively for SMA (Fig. 2A), vimentin (Fig. 2B), and the *SmoM2* transgene (Fig. 2I). They did not express CK19 (a cytokeratin expressed in the ductal cell lineage),

expression (anti-YFP, *red*) in mesenchymal tumor (MT) arising from a muscular blood vessel (CD31, *green*) in a 12-week-old *PdxCre;Kras^{G12D};SmoM2* mouse. (J) Hh signaling is active in mesenchymal tumor, as indicated by nuclear β -galactosidase enzymatic activity (*blue*) around blood vessel (CD31, *brown*). (K) SmoM2-YFP (*red*) expression in a higher-grade mPanIN epithelium (marked by CK19, *green*). (L) Hh signaling is not active in CK19-positive (*green*) neoplastic epithelium, as demonstrated by a lack of β -galactosidase immunoreactivity (*red*). β -galactosidase expression (*red*) is only observed in stromal cells adjacent to a higher-grade mPanIN (CK19, *green*; adjacent serial section to K). These images are representative of analysis of three animals per genotype. (Scale bars, 50 μ m.)

In contrast to early reports of Hh pathway activity in pancreatic tumor cells (13), we have recently shown in xenograft models a paracrine requirement for the Hh pathway, where Hh ligand is produced by the tumor cells and the pathway is activated in tumor stroma (18). To address whether a paracrine Hh signal is present in autochthonous mouse pancreatic tumors, and to test if tumor epithelium is competent to transduce the Hh signal, we used an oncogenic form of Smo to activate the pathway cell autonomously. This approach was successfully used to study cell-autonomous Hh pathway activation in adult neural stem cells, cerebellum granule cell precursors, and neural crest progenitor differentiation (21, 22,

Fig. 4. Quantitative RT-PCR analysis of Hh pathway components in PDA and colorectal tumors. (A) RT-PCR analysis of Hh pathway genes in the stromal vs. epithelial compartments following LCM of pancreatic samples from various mouse models. Relative expression of Hh pathway genes is displayed as $2^{-\Delta\Delta Ct}$ in the stroma vs. epithelium. CK19 shows the expected epithelium marker enrichment within the epithelial compartment. (B) RT-PCR analysis of Hh pathway genes in the stromal vs. epithelial compartment of human pancreatic carcinoma samples. *GLI1* is up-regulated up to 120-fold in the stroma compared to the epithelium. Both *SHH* and *IHH* are enriched in the epithelium (up to 10-fold) when compared with the stroma. (C) RT-PCR analysis of Hh pathway genes in the stromal vs. epithelial compartments of liver metastases from human colorectal cancers.

29). Expression of SmoM2 alone within pancreatic epithelium did not lead to significant pathology by 18 months of age. In addition, SmoM2 in combination with *Kras*^{G12D} does not accelerate *Kras*^{G12D}-driven pancreatic adenocarcinoma progression. The finding that SmoM2 could not activate Hh signaling in normal, metaplastic, premalignant, or malignant exocrine pancreatic epithelia in vivo, and in pancreatic ductal epithelial cells in vitro, indicates that epithelia of the exocrine pancreas are not competent to respond to Hh signals. Interestingly, correct localization of SmoM2 was detected in primary cilia of both ductal and islet epithelium, which is required for pathway activation (30). These results indicate a functional block of signaling downstream of Smo within the pancreatic epithelium in vivo.

The expression of Hh ligands by the tumor epithelium and the restriction of *Ptc-LacZ* in several mouse models of PDA to sur-

rounding SMA-positive stromal cells support a paracrine mechanism of Hh activation. Our finding that Hh signaling does not occur in tumor epithelium is also consistent with recent results showing that genetic ablation of Smo in the pancreatic epithelium of *p48-Cre/+;LSL-Kras*^{G12D}; *Trp-53*^{F/+} mice does not affect PDA tumor progression (31). The mechanism by which Hh pathway activation in stromal cells supports tumor growth remains to be established. The spindle-shape and SMA expression of *Ptc-lacZ*-expressing cells within mouse PDA suggests that many Hh-receptive cells in the tumor stroma are myofibroblasts. Consistent with this, cultured primary mouse pancreatic fibroblasts up-regulate the expression of secreted growth factors, such as Igf and Pdgf, following Hh stimulation (data not shown). These factors are similar to those regulated in subcutaneous xenograft models of PDA upon treatment with Hh pathway inhibitors (18).

The expression profiles of both the stromal and epithelial compartments of human pancreatic and metastatic colorectal tumors reported here support a model where activation of the Hh pathway is restricted to the stroma. The absence of mutations in Hh pathway components, such as PTCH, SMO, or SUFU in PDA is also consistent with the absence of canonical Hh pathway activation in tumor epithelium. Our findings that Hh signaling is restricted to the tumor stroma contradicts previous reports supporting a role for ligand-driven epithelial Hh signaling in tumor cell growth. While these conclusions were mostly derived from studies carried out in vitro, using tumor cell lines and cyclopamine at concentrations in vast excess to what is needed to inhibit Hh signaling (18), a recent mouse model has argued in favor of a role for Hh signaling in tumor epithelium. By expressing a dominant active *Gli2* (*CLEG2*), in pancreatic epithelium using a *Pdx* promoter, both *Kras*^{G12D}-driven PDA progression and the development of undifferentiated carcinoma was seen (14). However, this strategy does not reflect ligand-mediated activation of the canonical Hh pathway, as does SmoM2. *CLEG2*-expressing mice may develop pancreatic tumors because this dominant active *Gli2* allele is downstream or resistant to key posttranslational regulators of canonical Hh signaling. Post-translational regulators downstream of Smo have been shown previously to be key determinants in the temporal and spatial control of Hh target-gene expression in tumors (32). In another study, mutations in the *GLI* transcription factors were identified in human PDA tumor epithelium (33). While this leaves open the possibility that pancreatic epithelial *GLI* activity contributes to PDA tumorigenesis, the functional significance of the reported *GLI* mutations remains to be determined. Our study does not rule out the possibility that *GLI* activation plays a role in some PDA tumors, but shows that this activation is not the result of canonical Hh pathway signaling induced by Hh ligand over-expression. Interestingly, *Gli* activation in response to TGF β signaling has recently been reported in PDA (31), suggesting that other signaling pathways may be using the *Gli* transcription factors.

While the Hh signaling pathway is not active in tumor cells, its effect in tumor stroma may offer new avenues for the treatment of PDA, a disease often associated with abundant fibrosis and desmoplastic stroma (34, 35). This new paracrine model of Hh-mediated tumorigenesis may have important therapeutic consequences. Inhibition of a pathway active in tumor stroma may target cells and tumor-promoting mechanisms that are currently not affected by other cytotoxic agents that target the tumor epithelium, and may therefore be effectively used in combination with such agents.

Materials and Methods

Mice. *R26-SmoM2* (referred to as *SmoM2* allele in the present study) was kindly provided by Andrew McMahon before publication. *PdxCre* transgenic mice and *LSL-Kras*^{G12D} mice (2) (referred as *Kras*^{G12D} allele) were obtained from Andrew Lowy, University of Cincinnati, OH. *Ink4a/Arf*^{flax/flax} was provided by Anton Berns from the Netherlands Cancer Institute. *P53*^{R270H} and *LSL-Kras*^{G12D}