Pancreatic Cancer and Hedgehog Pathway Signaling: New Insights

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Abstract

While several aberrant signaling pathways have been attributed to the formation and progression of pancreatic cancer, there is mounting evidence for the increased role of the Hedgehog (Hh) pathway in multiple aspects of pancreatic tumor development. The Hh pathway is a signaling cascade that plays an important role in cell patterning of multiple tissues and organs, including the development of the gastrointestinal system. While normal pancreatic tissue exhibits little Hh pathway activity, patients with pancreatic adenocarcinoma show high levels of Hh pathway signaling in both the tumor epithelia and the surrounding mesenchyme. Several recent studies have focused on this paracrine activation of Hh signaling in the tumor microenvironment and have provided evidence for how activation of this pathway may play roles in mediating cellular proliferation, metastasis, and resistance to therapy. Together, these findings present new insights into how modulation of this pathway may allow us to target multiple aspects of pancreatic tumor biology.

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Introduction

The hedgehog pathway (Hh) plays a critical role during the development and specification of embryonic tissues and organs. Active signaling is stimulated by three known ligands: Sonic hedgehog (SHh), Indian hedgehog, and Desert hedgehog. In the absence of a ligand, the Hh ligand receptor Patched1 (PTCH1), which is located on the plasma membrane, represses the activity of the transmembrane protein Smoothened (SMO) through a mechanism still not clearly understood (fig. 1). In this condition, the GLI transcription factors, specifically GLI3, are phosphorylated and processed to a repressor form. Conversely, ligand binding to PTCH1 causes the internalization of the receptor and activation of SMO. A signaling cascade downstream of SMO leads to processing of the GLI transcription factors as activators, thus activating transcription of downstream target genes [1]. Interestingly, among the best characterized target genes are components of the pathway itself, including PTCH1, GLI1 and HIP. This ensures that the activity level of the Hh pathway is tightly regulated through a feedback mechanism. Depending on the cellular context, other downstream targets include cell proliferation and differentiation factors like Wnt proteins, TGF β , and FGF pathway components, as well as cell cycle regulators like p21 and cyclin D1 [2].

Fig. 1. Diagram of canonical Hh pathway activation. **A** In the absence of ligand GLI transcription factors are downregulated or processed to a repressor form which prevents activation of downstream target genes. **B** Following ligand binding SMO releases the repression on GLI transcription factors and they translocate to the nucleus to activate downstream target genes.

Activity of the Hh signaling pathway is required during the embryonic development of the gastrointestinal tract, with the notable exception of the pancreas, where Hh activity is repressed by activin β B and FGF2 signals released by the notochord [3]. This down-regulation of the Hh pathway is critical for pancreatic development, as forced expression of SHh in the pancreatic anlage results in agenesis of the pancreas in mouse embryos [4], while repression of Hh signaling in areas of the developing gut that normally express it results in ectopic expression of pancreas-specific genes in the stomach and intestine [5]. This quiescent state of the Hh pathway in the pancreas continues into adulthood. In the adult pancreas, the Hh pathway activity is typically very low; however, it can be activated under circumstances such as injury or disease [6].

Hedgehog Pathway Activation in Pancreatic Cancer

Pancreatic ductal adenocarcinoma is the most common and lethal form of pancreatic cancer. While the cell of origin of pancreatic adenocarcinoma is still a matter of debate, there is general consensus on a progression model for this disease. The most common precursor lesions of pancreatic cancer are known as PanINs (pancreatic intraepithelial neoplasias) and they are classified from 1A to 3 (the latter representing carcinoma in situ) based on

defined histological characteristics [7]. Genetic alterations in PanIN lesions and pancreatic cancer have been the subject of numerous studies. The defining mutation of human pancreatic cancer which is found in greater than 80–90% of pancreatic cancers is a single amino acid change in the K-ras gene, often in codon 12 or 13 that generates a constitutively active form of the protein [8, 9]. The Ras signaling pathway includes a number of GTPases that control signaling for many important cell functions, including proliferation, cell migration, adhesion, and apoptosis. This mutation has already been identified in the earliest precursor lesions of pancreatic cancer, known as PanIN1A. Other common mutations, usually observed at a later stage of disease progression, are loss of tumor suppressor genes such as p53, p16^{Ink4a} and DPC4, a component of the TGF β signaling pathway [10].

Activation of the Hh pathway in primary human pancreatic cancer was first reported in two parallel studies [11, 12]. The pathway was found to be activated in about 70% of human pancreatic cancer cases as well as in the majority of pancreatic cancer cell lines. Pathway activation was driven by over-expression of Hh ligands, mainly SHh and Indian hedgehog [12]. A similar mechanism of pathway activation has been described for prostate [13] and lung cancer [14]; however, it differs from the mutation-driven activation of the Hh signaling pathway that has been well characterized in basal cell carcinoma and medulloblastoma [15, 16]. Surprisingly, however, a recent

Fig. 2. Model of Hh signaling in the tumor microenvironment. Tumor cells release high concentrations of SHh ligand into the tumor space and activate Hh signaling in the surrounding stromal compartment. Activation of the Hh pathway in stromal cells presumably leads to the expression of still unknown factors that contribute to tumor growth and metastasis. The desmoplastic reaction due to the expansion of activated stellate cells may also recruit inflammatory cells that also contribute to the support of the tumor.

comprehensive mutational study of pancreatic cancer led to the identification of mutations in components of the Hh pathway in the majority of the cases tested [17]. Further studies will be required to better characterize those mutations and assess whether or not they confer aberrant signaling of the pathway.

A deeper insight on the role of Hh signaling in pancreatic cancer and its relationship with other oncogenic pathways has been obtained through the study of mouse models of this disease. The most representative mouse models of pancreatic cancer are based on the expression of a mutated form of the K-ras gene which mimics what is found in primary human cancers, specifically in the pancreatic epithelium [18, 19]. In the transgenic animals, PanIN lesion development and progression closely resembles the human disease and, interestingly, exhibits expression of the SHh ligand in the majority of mouse PanINs, even in very early developing lesions. Similar activation of the Hh pathway following initiation of the disease using K-ras mutation has been observed, even in a recently described zebrafish model of pancreatic cancer [20].

In these disease models, Hh pathway activation is downstream of K-ras signaling (fig. 2). Insight into the mechanism of Hh activation by oncogenic K-ras has been

provided by studies indicating that SHh might be a downstream target of NF- κ B [21]. This pathway is up-regulated in response to inflammatory stimuli and cellular stress, conditions found in the wounding environment of a neoplastic lesion. Detailed study of the SHh promoter and upstream region revealed multiple NF- κ B binding sites that were able to activate transcriptional activity of SHh in both in vitro and in vivo models [22]. This study implies that NF- κ B serves as a link between oncogenic K-ras and Hh signaling.

Other mouse models have addressed the role of Hh signaling in pancreatic cancer more directly. Initial examination of the role of Hh signaling in the pancreas was carried out in transgenic animals expressing SHh in the pancreatic epithelia using the PDX1 promoter that drives expression to the early pancreatic epithelium. These animals displayed lesions that closely resembled human PanINs and showed elevated expression of HER2/neu and mutated K-ras typically seen in pancreatic cancers [11]. However, it is not clear whether or not these animals would progress to a more advanced phenotype because ectopic expression of the SHh ligand in the pancreatic epithelium disrupted normal pancreas development and these animals died at an early age due to pancreatic developmental defects. In an alternative approach, an active

form of the transcription factor GLI2 was expressed specifically in the pancreatic epithelium starting from early pancreatic development [23]. Epithelial expression of the GLI transgene did not disrupt pancreas development, but it did cause formation of pancreatic tumors described as undifferentiated carcinomas in adult animals. Those tumors did not progress through PanINs and bear little resemblance to human pancreatic ductal adenocarcinoma. However, simultaneous expression of active GLI2 and mutant K-ras resulted in early onset of PanINs, indicating a synergy between activation of K-ras and Hh in PanIN progression. Interestingly, these studies have also shown a correlation between expression of SHh and expansion of the mesenchymal compartment surrounding the epithelial cells, thus indicating a possible role of SHh in promoting the desmoplastic reaction in pancreatic cancer. Altogether, these results suggest a scenario in which spontaneous K-ras mutations lead to PanIN lesion formation and, in turn, the resulting inflammatory reaction leads to the up-regulation of the Hh pathway. Further accumulation or 'hits' including loss of p53 or p16^{INK4a} tumor suppressors lead to a more aggressive phenotype as other studies using primary cell culture model systems have indicated [24].

Effects of Hedgehog Pathway Activation in the Tumor Microenvironment

One of the characteristic features of pancreatic adenocarcinoma is the level of desmoplasia found in the diseased tissue. This desmoplastic reaction results from the activation and uncontrolled growth of supporting cell types including fibroblasts, stellate cells, immune cells, and vascular-associated cells [25]. In the context of the tumor microenvironment, expansion of these cells creates a growth-rich environment along with a physical barrier that shields the tumor cells from responding to pharmacological treatments [26, 27].

While several studies have shown robust expression of SHh ligand in the tumor epithelium, it is unclear how cells in the surrounding microenvironment respond to these signals. Recent studies suggest that stromal cells in the pancreatic tissue are Hh responsive and can signal the expansion of the stromal compartment [28]. Transformed pancreatic epithelial cells with over-expression of the SHh ligand implanted orthotopically into nude mice will signal expansion of stromal cells as evaluated by staining with the differentiated fibroblast marker α -smooth muscle actin, and can be modulated with treatment of ani-

mals with a blocking peptide that inhibits Hh signaling. Additionally, expression levels of extracellular matrix proteins, which are characteristic of the desmoplastic reaction in pancreatic cancer, are significantly higher in SHh expressing tumors compared to tumors lacking SHh over-expression.

Further evidence supporting a paracrine role for Hh signaling in pancreatic tumors was shown in the treatment of primary pancreatic tumor xenografts with a novel Hh pathway inhibitor which exhibited specific down-regulation of Hh pathway genes in the mouse stromal compartment compared to the human tumor cells in which Hh pathway genes were unchanged [29]. Paracrine Hh signaling was confirmed in these studies by transplanting tumor cells with either high or low expression of SHh into transgenic *Ptch1-LacZ Rag2*^{-/-} animals that are able to accept human cell grafts and will read-out Hh signaling by positive β -galactosidase expression. Tumors with high SHh expression displayed robust β -galactosidase staining in the stromal compartment adjacent to the tumor cells, while the tumors with low SHh exhibited little or no expression of β -galactosidase. Treatment of these tumors with a Hh pathway inhibitor (HhAntag) resulted in significant decreases in tumor growth, but following evaluation of the tumors by qRT-PCR, using species-specific mouse/human primers to gauge the effect in both compartments, only the mouse stromal cells showed down-regulation of Hh pathway genes. This suggests that tumor growth was inhibited not by targeting the tumor cells, but by disrupting the pancreatic tumor microenvironment. Perhaps the most intriguing data is the almost complete abrogation of tumor growth by co-transplanting SHh-positive human tumor cells with mouse embryonic fibroblasts that have been rendered unresponsive to Hh signaling by knockout of the Hh signaling receptor smoothed (SMO). Tumors implanted with the SMO knockout mouse embryonic fibroblasts were shown to be much smaller or did not form at all compared to the large tumors that formed within the same time frame as the tumor cells implanted with wild-type SMO fibroblasts. While this study confirms the importance of the Hh pathway for pancreatic cancer growth, it also opens a debate on its relative importance in the epithelial compartment and in the tumor stroma.

Our current understanding indicates that the SHh ligand produced by the tumor epithelial cells activates the 'canonical' Hh pathway in the surrounding fibroblasts through a paracrine mechanism [29]. At the same time, GLI transcription factors are expressed and activated in the pancreatic epithelium [11]; however, their activation

in the epithelium may not be mediated by the Hh pathway, but by an alternative mechanism involving K-ras and the TGF β pathway [30, 31]. Regardless, GLI activity in the epithelial cells is required for their proliferation and survival. More studies will be required to dissect the function of the Hh pathway in the different cell compartments, including the epithelial and mesenchymal, and even in inflammatory cells [32], which may play an important role as well. Moreover, further insight is needed in the downstream effectors of Hh signaling in pancreatic cancer to better determine whether different subsets of GLI target genes are activated in the different cell compartments (fig. 2).

Hedgehog Pathway and Cancer Stem Cells

A shifting paradigm in how we view cancer is the discovery that tumors are comprised of a heterogeneous mixture of cells with distinct populations that have unique tumor-initiation capability, termed cancer stem cells. Much of the groundwork for identifying these cells was initiated by the application of lessons and techniques learned in identifying populations of normal, nontumorigenic stem cells. These assays included the use of cell surface marker staining by fluorescent-activated cell sorting and the use of immune-compromised animal host models. To assess for these tumor-initiating cells, primary human tumors were isolated and single cell suspensions from these cancers were stained with various cell surface marker combinations and then implanted orthotopically or subcutaneously in immune-compromised animals. The resulting tumors were analyzed for their ability to recapitulate the characteristics of the primary tumor as well as serially transplanted into recipient animals to assay for self-renewal. This has resulted in the identification of several cancer stem cell populations, initially from blood-borne cancers [33] and then in solid tumors of the breast [34], brain [35], colon [36], and several other solid tumor systems. These concepts have been met with some controversy as others have challenged that these cancer stem cells are the result of artifacts of the assay and, given the proper conditions, the 'differentiated' cancer cell population could form tumors in animals. It has even been argued that these tumor-initiating cells are not as rare as we think [37]. While much work is still needed to answer these questions in all tumor model systems, evidence from the identification of breast cancer stem cells in mouse models of breast cancer which bypass concerns about human/mouse xenograft models [38],

along with data describing unique abilities of cancer stem cells to evade radiation [39] and chemotherapy treatments [40], make it clear that these are important cells to study and target in the treatment of patients.

Evidence for a role of Hh signaling in cancer stem cells has come from both hematopoietic and solid tumor models of cancer. Specifically in breast cancer stem cell populations, these cells were found to have a 30-fold higher level of expression of GLI1 along with significant up-regulation of PTCH1 and GLI2 compared to nontumorigenic cells [41]. Additionally, in human glioma cancer stem cells, Hh pathway activation has been shown to be vital to the growth and survival of these cells, and down-regulation of GLI transcription factors, either by chemical or molecular inhibitors, leads to marked effects on cancer stem cell self-renewal and tumorigenicity [42].

In our own studies, we have identified a distinct population of cells within primary human pancreatic adenocarcinoma that are enriched in tumor-initiating cells and exhibit self-renewal by serial passaging in NOD/SCID animals [43]. These cells, which are marked by the expression of the cell surface markers CD44, CD24, and epithelial surface antigen, represent a rare, yet self-renewing population of tumorigenic cells which can establish tumors phenotypically identical to the primary patient tumor. Interestingly, direct isolation and qRT-PCR of these cells revealed higher levels of SHh expression compared to the nontumorigenic population. We are currently exploring the role of the hedgehog signaling pathway in pancreatic cancer stem cell function derived from primary pancreatic cancers. Others have indirectly tested the importance of Hh signaling in pancreatic cancer stem cells by treatment of a metastatic cell line model of pancreatic cancer with the SMO inhibitor, cyclopamine [44]. These treatments did not significantly alter tumor size, but exhibited a significant effect on preventing tumor metastasis and led to a decrease of the cancer stem cell population as assessed by a reduction in aldehyde dehydrogenase activity, a marker used to identify tumorigenic populations in the breast [45], colon [46] along with normal stem cells in several tissue types. These effects may also be mediated by blockage of Hh signaling in the tumor stroma and inhibiting cross-talk that may be vital to pancreatic cancer stem cell maintenance in light of the more recent data describing only a paracrine requirement for Hh signaling in pancreatic cancer [29]. These will be important questions to answer in the future as we develop better in vitro and in vivo models that allow us to construct a more comprehensive picture of the role of Hh pathway signaling in the tumor microenvironment.

Conclusions and Future Directions

Activation of Hh signaling is clearly an important early event in pancreatic cancer formation as well as promoting tumor growth and metastatic potential. Recent work demonstrating that Hh signaling is activated in the pancreatic tumor stroma has yielded further insight on how we may use Hh pathway inhibitors to target therapeutic

treatments. An exciting recent development is the identification of pancreatic cancer stem cells and data suggesting that Hh signaling may be important in regulating their function. Further studies in elucidating the role of this signaling pathway are likely to yield important information that ultimately may improve therapeutic approaches and subsequently improve outcomes in patients with pancreatic cancer.

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