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Extra View

Shh Signaling and Pancreatic Cancer

Implications for Therapy?

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ABSTRACT

Hedgehog signaling has been implicated in the development of several human cancers, including small cell lung carcinomas, medulloblastomas, basal cell carcinomas, and digestive tract tumors. Elevated levels of pathway components are observed in pancreatic ductal adenocarcinoma (PDAC) precursor lesions, and these levels increase further as lesions progress to more advanced stages. Yet the mechanisms by which hedgehog signaling contributes to pancreatic tumorigenesis were poorly understood. We recently published results showing that activated hedgehog signaling enhances the proliferation and survival of pancreatic duct epithelial cells, the presumptive target cells for PDAC development. We also demonstrated that sonic hedgehog (Shh) expression, in cooperation with loss of the Trp53 and Ink4a/Arf tumor suppressor loci, was sufficient to initiate the formation of early pancreatic lesions. Furthermore, Shh signaling enhanced K-Ras-mediated pancreatic tumorigenesis and reduced the dependence of tumor cells on the sustained activation of Ras-stimulated signaling pathways. Here we discuss the significance of these findings and the implications for therapy.

INTRODUCTION

The hedgehog signaling pathway is vital for embryonic development, particularly gastrointestinal patterning.1,2 Shh is also active in a subset of cells in mature organs and may play a role in maintaining stem cell number and accurate patterning in the epithelia of the lungs, the skin3 and the digestive tract.4,5 Deregulation of hedgehog signaling has also been observed in several human cancers, including small cell lung carcinomas, medulloblastomas, basal cell carcinomas and digestive tract tumors.4,7 In fact, activation of the hedgehog signaling pathway occurs in a majority of pancreatic ductal adenocarcinomas.4,5

There are three mammalian hedgehog genes: Sonic (Shh), Indian (Ihh) and Desert (Dhh), all of which encode signaling molecules that undergo autocatalytic cleavage and double lipid modification to generate an active ligand.2,8 In the absence of hedgehog ligand, the hedgehog receptors, Patched1 and Patched2 (hereafter denoted as Ptc), are involved in repression of the hedgehog signaling molecule Smoothened (Smo).9 Upon ligand binding, Smo is released from inhibition, providing a signal for the dissociation of Gli transcription factors from an inhibitory complex that includes the serine/threonine protein kinase Fused (Fu), and Suppressor of Fused [Su (Fu)].9 The Gli transcription factors translocate to the nucleus where they regulate the transcription of hedgehog responsive genes including Ptc and Gli itself.10 Also among the reported targets of hedgehog signaling are the genes encoding the cell cycle regulators Cyclin D1, N-Myc and p21, and the Wnt proteins.5,11,12

Pancreatic cancer is a very aggressive malignancy, exemplified by a five year survival rate of 5% and median survival of less than six months.13,14 Approximately 30,000 Americans are diagnosed with pancreatic cancer each year, and an equal number die from the disease, making this malignancy the fourth leading cause of cancer-related deaths in the United States.14 Pancreatic cancers arise from the three major cell types within the organ - acinar cells, endocrine cells and duct epithelial cells, however pancreatic ductal adenocarcinoma (PDAC) accounts for more than 85% of all cases.15 The putative target cells of PDAC are the duct epithelial cells, although the exact nature of the progenitor cell has not been identified.10 PDAC arises from precursor lesions called pancreatic intraepithelial neoplasms (PanINs). These lesions sequentially acquire specific genetic alterations during progression towards malignancy, including activation of K-Ras and loss of Ink4a in PanIN
1 and 2, loss of p53 in PanIN 2–3, and loss of Smad4 in PanIN 3.17 PanIN lesions are also characterized by specific histological changes. These include conversion of the normal cuboidal duct epithelial cells to a columnar phenotype, formation of papillary architecture and mucin accumulation in PanIN 1, loss of polarity and appearance of atypical nuclei in PanIN 2, and luminal budding and increased mitoses in PanIN 3.17,18

Intriguingly, Shh is excluded from the developing pancreas, as well as the mature organ,19 yet is expressed in early PanIN lesions, with increasing levels as lesions progress to invasive PDAC.5 Ectopic expression of Shh under the control of the Pdx-1 promoter, active in pancreas progenitor cells, leads to ductal abnormalities accompanied by mutations in K-Ras - an early event in PDAC development.5 Shh signaling is also active in a majority of pancreatic cancer cell lines, and inhibition of hedgehog signaling blocks proliferation and induces apoptosis in a subset of these cell lines, both in vitro and in vivo.5 Further, a study of the gene expression profiles of early PanIN lesions revealed upregulation of several foregut markers, many of which were also upregulated in Gli1 overexpressing human pancreatic duct epithelial cells.20 Thus, there is growing evidence to suggest that activated Shh signaling is a critical early mediator of pancreatic cancer development.

**Shh CONTRIBUTES TO PANCREATIC TUMOR INITIATION**

Given this information we investigated the mechanisms by which Shh contributes to pancreatic tumorigenesis. We found that Shh expression enhances the proliferation of pancreatic duct epithelial cells, potentially through the transcriptional regulation of the cell cycle regulators cyclin D1 and p21.21 We also observed increased phosphorylation of the signaling molecules Akt and Erk1/2 in cells with active Shh signaling.21 Activation of the PI3K/Akt pathway is implicated in enhancing cell survival, while Erk1/2 activation is associated with cell proliferation. Collectively, these data indicate that Shh stimulates proliferation of PDECs through the regulation of multiple molecules. We also found that in addition to providing a proliferative stimulus, Shh expression confers protection from death receptor-dependent, caspase 8-mediated apoptosis, at least partially through post-transcriptional activation of Bcl-2 and Bcl-XL.21 Orthotopic transplantation of PDECs with constitutive activation of Shh signaling and loss of the Ink4a/Arf or Trp53 tumor suppressor loci, was sufficient to induce the formation of early pancreatic ductal lesions, but not pancreatic carcinomas.21 This result is consistent with previous findings that Shh pathway activation is present within early PanIN lesions, and supports a role for the Shh pathway in PDAC initiation.

It remains unknown how Shh signaling activates the MAPK and Akt/mTOR pathways in PDECs, however, previous studies have demonstrated that PDGF,22,24 and IGF signaling components24 are targets of Shh signaling. Thus, one likely possibility is that Shh stimulates receptor tyrosine kinase signaling, resulting in activation of MAPK and PI3K/Akt/mTOR pathways. Interestingly, we, and others, have shown that expression of activated Gli molecules does not result in the stimulation of Erk.21,25 Intriguingly, while we did not observe activation of Akt in PDECs expressing an activated Gli1 molecule, Pasca di Magliano et al23 observed Akt phosphorylation in pancreatic tumors induced by an activated Gli2 allele. This discrepancy may reflect functional differences between Gli1 and Gli2, or it may indicate a requirement for PI3K/Akt signaling in pancreatic tumor development, which occurred via a Gli2-independent mechanism. Analysis of PDECs from mice expressing the activated Gli2 allele might address this question. Thus, the data indicate that Shh may signal at least in part through Gli-independent mechanisms to activate Akt and Erk.

**Shh AND FAS SIGNALING IN PANCREATIC CANCER**

In order to progress, pancreatic tumors must escape immune clearance by tumor specific cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. A major pathway responsible for CTL and NK cell mediated apoptosis is the Fas-Fas ligand (FasL) system. When CTLs or NK cells recognize target cells, they become activated and express FasL, which binds to Fas receptors on the surface of target cells and induces their apoptosis.20 Cancer cells frequently display decreased sensitivity to apoptotic stimuli, and previous studies have linked Shh signaling to cell survival in some experimental systems. We found that Shh expression protects PDECs from a caspase 8- and caspase 3-dependent apoptotic pathway (Fig. 1A). This protective effect of Shh appears to be mediated in part through the stabilization of the anti-apoptotic proteins Bcl-2 and Bcl-XL. This is consistent with previous observations that Hedgehog signaling can enhance apoptosis in a subset of these cell lines, both in vitro and in vivo.5 Further, a study of the gene expression profiles of early PanIN lesions revealed upregulation of several foregut markers, many of which were also upregulated in Gli1 overexpressing human pancreatic duct epithelial cells.20 Thus, there is growing evidence to suggest that activated Shh signaling is a critical early mediator of pancreatic cancer development.

**Figure 1.** Shh protects PDECs from Fas-induced apoptosis. (A) Trp53 null, Ink4a/Arf null PDECs infected with either Shh or GFP were plated out at a density of 1 x 10^4 cells per well, and allowed to attach. Cells were pretreated with inhibitors of caspase 3 or 8 (20 μM) for 1 hour, as indicated, then treated with 100 μM cycloheximide, or vehicle for a period of 24 hours. (B) Trp53 null, Ink4a/Arf null PDECs and infected with either Shh GFP were plated out at a density of 1 x 10^4 cells per well, and allowed to attach. Cells were pretreated for 1 hour with anti-Fas, antibody (MFL3) as indicated, and then treated with 100 μM cycloheximide, or vehicle for a period of 24 hours. Cells were harvested and counted and viability assessed by trypan blue exclusion.
levels of Bcl-2 in basal cell carcinoma cell lines and medulloblastoma cells, although in these studies transcriptional activation of Bcl-2 was observed.27-29 Activation of caspases 8 and 3 is associated with death receptor-induced apoptosis,30 and previous data have shown that Shh expression is able to rescue cells from apoptosis mediated by the Fas death receptor.31 Furthermore, apoptosis mediated by the Shh pathway inhibitor cyclopamine, requires Fas: FasL interaction,32 and cyclopamine treatment upregulates Fas expression in BCC cell lines, while expression of activated Smo inhibits Fas expression in these cells.32 We showed using blocking antibodies, that inhibiting Fas: FasL interaction protects PDECs from apoptosis (Fig. 1B), implicating the Fas pathway in apoptosis in PDECs, and indicating a role for Shh in protecting PDECs from death receptor mediated apoptosis.

Our findings are of particular interest given the potential role of Fas signaling in pancreatic cancer. Prior studies have shown that pancreatic cancer cells express the death receptors Fas, TRAIL and TNF-R but are strongly resistant to death receptor-induced apoptosis, possibly due to over-expression of the anti-apoptotic proteins Bcl-2 and Bcl-XL.33 This is also interesting in light of our recent finding that Shh signaling in pancreatic duct cells can lead to stabilization of Bcl-2 and Bcl-XL in response to apoptotic stimuli.21

Previous work has shown that many pancreatic cancers and pancreatic cancer cell lines lose expression or function of Fas,34,35 and the loss of Fas has been shown to correlate with extra-pancreatic spread and shorter overall survival in PDAC patients.35 Perhaps more intriguingly, Fas ligand is frequently expressed in human PDAC and pancreatic cancer cell lines, but is not expressed in the normal adult pancreas.24 This combination of events may provide PDACs with a degree of protection from the immune response. FasL is expressed not only by immune cells, but also on nonlymphoid cells in organs where an inflammatory reaction might cause damage, for example the eyes, brain and testes.36,37 In these organs, cell surface FasL expression induces apoptosis in infiltrating pro-inflammatory cells.36,37

Several studies have now shown that certain tumors may also be sites of immune privilege.38 A variety of cancer cell lines have been shown to induce apoptosis in Fas-expressing lymphoid cells in vitro, and there is growing evidence to suggest that a similar situation may exist in vivo.38 For example, down-regulation of FasL expression in colon tumor cells significantly reduced tumor development in syngeneic immunocompetent mice, and led to increased lymphocyte infiltration.39 Our data raise the possibility that Shh signaling can render pancreatic cancer cells insensitive to Fas-mediated apoptosis, thus allowing protection from infiltrating T cells, and enabling a counter-attack against tumor-reactive immune cells (Fig. 2). If this is the case, immune based therapeutic strategies such as adoptive T cell therapy or cancer vaccines would not be predicted to be efficacious. Novel approaches to therapeutic intervention might aim to neutralize this counterattack or re-establish tumor cell sensitivity to Fas.

### Shh AND K-RAS IN PANCREATIC TUMOR FORMATION AND PROGRESSION

Activating K-Ras mutations are one of the most frequent genetic alterations associated with pancreatic cancers, detected in over 90% of all pancreatic adenocarcinomas. We found that orthotopic transplantation of K-Ras-infected PDECs lacking either Ink4a/Arf or Ink4a/Arf and Trp53 leads to the development of undifferentiated carcinomas within sixty days of transplant.21 This is in contrast to our finding that transplantation of Shh-infected PDECs induces early atypical ductal lesions within the pancreas that fail to progress further within 120 days.21 However in vitro, Shh stimulates proliferation to a similar extent as activated K-Ras in cells lacking the Trp53 and Ink4a/Arf tumor suppressor loci.21

This enhanced ability of activated K-Ras, compared with Shh, to transform PDECs is of great interest given that cells of each genotype proliferate at a similar rate, and exhibit activation of the signaling molecules Akt and Erk1/2. The increased capacity for transformation by K-Ras may reflect an enhanced survival advantage. Alternatively, it may reflect the activation of the Ral signaling pathway by K-Ras, but not Shh, as previous studies in other experimental systems have shown that activation of the Ral signaling pathway principally mediates the transforming properties of activated Ras proteins.40,41 Analysis of the activation status of this, and other, signaling pathways in Ras-expressing PDECs compared with Shh-expressing PDECs should provide additional insights into the mechanisms important for pancreatic cancer development, and further, may identify potential therapeutic targets.

However, we have found that Shh cooperates with activated K-Ras in the initiation and maintenance of pancreatic tumors.21 Shh enhanced tumor initiation by K-Ras, and increased tumor volume. Similar findings were made in a model of PDAC induced by a constitutively active Gli2 allele.25 Investigation of cell lines isolated from tumors induced by either K-Ras alone or Shh and K-Ras revealed that cells derived from K-Ras-expressing tumors were highly sensitive to inhibition of the MAP kinase and Akt/mTOR signaling pathways, while cell lines expressing both K-Ras and Shh continued to proliferate despite inhibition of these signaling pathways.21 Furthermore, simultaneous inhibition of these pathways failed to induce complete arrest in cells expressing K-Ras and Shh. However, Shh pathway inhibition, using either cyclopamine or Smo-targeting shRNAs, coupled with PI3K pathway inhibition led to growth arrest and cell death. Thus, these data indicate that Shh signaling in pancreatic cancer cells can reduce the requirement of tumor cells for oncogenic Ras.
In Ras-induced tumors, low level Shh pathway activation must occur at the level of smoothened, or further upstream, since cell lines from these tumors are sensitive to inhibition of this molecule. However, cell lines from Ras-induced tumors with additional Shh signaling are less sensitive to pathway inhibition. This observation raises the possibility that Shh pathway activation may be qualitatively different in cells with Ras-activated Shh signaling, compared with cells ectopically expressing Shh. In addition, we cannot discount the possibility that Ras signaling also affects the Shh pathway further downstream. In fact, new evidence suggests that in cancer cells, MAPK and Akt signaling may regulate the nuclear localization and transcriptional activity of Gli.42 While in cultured fibroblasts Akt activation is able to potentiate Gli activation by low level Shh signaling,43,44 we have shown that Shh can stimulate the MAPK and PI3K/Akt pathways. Perhaps a positive feedback loop exists by which Shh signaling stimulates the MAPK and Akt pathways, while MAPK and Akt signaling can also stimulate Shh signaling at the level of the Gli transcription factors, reinforcing the activation of the pathway (Fig. 3). Thus, the K-Ras and Shh signaling pathways may synergize during pancreatic tumorigenesis in vivo.

The data demonstrate the sustained requirement for multiple Ras signaling pathways in tumor maintenance. This is in contrast with previous studies that have suggested that Ras transformed cells are dependent only on the sustained activation of the PI3K/Akt pathway.41 These findings also suggest that constitutive Shh pathway activation can reduce the dependence of tumor cells on activated K-Ras. This discovery is important since it suggests that pancreatic ductal tumors with activated Shh signaling will be resistant to inhibition of Ras-regulated signaling pathways. Pharmacological inhibition of Ras signaling has been proposed as a therapeutic strategy for PDAC, and several drugs that target Ras-regulated signaling pathways are already in development and in clinical trials. Given our results, further investigation in vivo using different models induced by a variety of genetic lesions, including Shh signaling, may be required to identify the conditions under which these compounds will provide therapeutic benefit in PDAC.

Collectively, the above findings indicate that there is substantial cooperation and cross signaling between Ras and Shh pathways. Delineating how hedgehog signaling is activated in PDAC, and how Shh signaling and Ras signaling interact, is crucial before attempting to antagonize either pathway as a therapeutic strategy.

References