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Ectopic Overexpression of Sonic Hedgehog (Shh) Induces Stromal Expansion and Metaplasia in the Adult Murine Pancreas[1,2]

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Abstract

Ligand-dependent activation of the Hedgehog (Hh) signaling pathway has been implicated in both tumor initiation and metastasis of pancreatic ductal adenocarcinoma (PDAC). Prior studies in genetically engineered mouse models (GEMMs) have assessed the role of Hh signaling by cell autonomous expression of a constitutively active *Gli2* within epithelial cells. On the contrary, aberrant pathway reactivation in the human exocrine pancreas occurs principally as a consequence of Sonic Hh ligand (Shh) overexpression from epithelial cells. To recapitulate the cognate pathophysiology of Hh signaling observed in the human pancreas, we examined GEMM where Hh ligand is conditionally overexpressed within the mature exocrine pancreas using a tamoxifen-inducible Elastase-Cre promoter (Ela-CreERT2;LSL-m*Shh*). We also facilitated potential cell autonomous epithelial responsiveness to secreted Hh ligand by generating compound transgenic mice with concomitant expression of the Hh receptor Smoothened (Ela-CreERT2;LSL-m*Shh*;LSL-m*Smo*). Of interest, none of these mice developed intraductal precursor lesions or PDAC during the follow-up period of up to 12 months after tamoxifen induction. Instead, all animals demonstrated marked expansion of stromal cells, consistent with the previously described epithelial-to-stromal paracrine Hh signaling. Hh

responsiveness was mirrored by the expression of primary cilia within the expanded mesenchymal compartment and the absence within mature acinar cells. In the absence of cooperating mutations, Hh ligand overexpression in the mature exocrine pancreas is insufficient to induce neoplasia, even when epithelial cells coexpress the Smo receptor. This autochthonous model serves as a platform for studying epithelial stromal interactions in pancreatic carcinogenesis.

Neoplasia (2011) 13, 923–930

Introduction

Ductal adenocarcinoma of the pancreas (a.k.a. pancreatic cancer) is one of the most deadly of human malignancies to date and is associated with almost uniform lethality [1]. Accounting for an estimated 36,400 fatalities in the United States alone in 2010, pancreatic cancer represents the fourth most common cause of cancer-related mortality in the western world [2]. Moreover, approximately 80% of cases are diagnosed at locally advanced or metastatic tumor stages, usually precluding surgical resection and leaving patients without any curative therapeutic option. With an overall survival of less than 6 months and 5-year survival rates below 5%, the dismal overall prognosis of pancreatic cancer has not markedly improved during the past decades [1,3,4].

Multiple lines of evidence implicate the Hedgehog (Hh) signaling pathway playing a role in both pancreatic cancer initiation and progression [5,6], as well as representing a promising target for therapeutic intervention [7–13]. The basis for aberrant pathway activation in pancreatic cancer is usually not a consequence of oncogenic mutations within canonical Hh pathway components but rather secondary to endogenous overexpression of Hedgehog ligands by neoplastic epithelial cells [5,7,8,14]. Conversely, the nature of the Hh-receiving cells in the context of pancreatic cancer is less clearly defined. Various models have been proposed, including stimulation of "bulk" neoplastic epithelial cells in an autocrine or paracrine manner, maintenance of a putative subpopulation of neoplastic epithelial cells with enhanced tumorigenic potential (also referred to as "cancer stem cells" by some authors), and most recently, proliferative effects on stromal cells of mesenchymal origin within the tumor microenvironment, as well as combinations of these models [15,16].

Among the more powerful tools in understanding the mechanisms underlying pancreatic carcinogenesis has been the development of genetically engineered mouse models of pancreatic cancer [17–19]. In this present study, we describe a genetically engineered mouse model in which Sonic Hedgehog ligand (Shh) is overexpressed in the acinar cell compartment of adult murine pancreata. In contrast to prior reports that relied on the ectopic expression of a constitutively activated *Gli2* in the murine pancreas [20], we recapitulated "physiological" conditions by inducing endogenous Hedgehog ligand expression from the adult exocrine cells. In this model, we fail to observe evidence of intraductal neoplasia in the absence of cooperating mutations, such as oncogenic *Kras*. Instead, the Shh ligand–overexpressing murine pancreata demonstrate a striking expansion of the periacinar mesenchymal compartment, consistent with paracrine epithelial-stromal signaling. This model should serve as a platform for elucidating epithelial-stromal interactions in the context of exocrine pancreatic neoplasia and, further, for developing relevant examples of oncogenic cooperation with Hh signaling.

Materials and Methods

Generation of Pdx1-Cre;LSL-mShh, Elastase-CreERT2;LSL-mShh, and Elastase-CreERT2;LSL-mShh;LSL-mSmo Mouse Cohorts

Generation of LSL-m*Shh* mice has been described previously [21]. In brief, in this model, Cre-mediated recombination leads to excision of the lox-stop-lox (LSL) cassette and conditional expression of mShh ligand (Figure 1*A*). An identical strategy is used for driving expression of Smo receptor protein in the recombined cells of compound transgenic LSL-m*Shh*, LSL-m*Smo* mice. Mice interbred and reproduced readily with an even distribution of female-to-male ratio of approximately 1:1. The presence of a constitutive fluorescent protein expression in nonrecombined tissues (e.g., tail snips) enables rapid detection of the LSL-m*Shh* or LSL-m*Smo* allele, by green or red fluorescence, respectively. The "driver" *Pdx1*-Cre and *Elastase* (*Ela*)-CreERT2 mice have been previously described [22,23]. The presence of *Cre* or *Cre*ERT2 alleles, respectively, was determined by polymerase chain reaction analysis, as previously described [23,24].

Two different reporter mouse crosses were generated in our studies: the first, to confirm the robustness of Cre-mediated recombination in the pancreatic acinar compartment, we generated Ela-CreERT2;*Rosa*26R mice, which harbor a *beta-galactosidase* gene downstream of a ubiquitous promoter and a LSL cassette. Second, to demonstrate Hh pathway activity within specific pancreatic compartments, the Ela-CreERT2;LSL-m*Shh* mice were also crossed into a *Ptch-lacZ* background; in this latter strain, the *lacZ* allele is knocked into one of the *Ptch* loci, and the resulting beta-galactosidase activity (indicative of Hh pathway activity) can be visualized by X-Gal staining [24,25].

The following three cohorts of mice with pancreas-specific Shh ligand overexpression were generated for observation: *Pdx1*-cre;LSL-m*Shh*, Ela-CreERT2;LSL-m*Shh*, and Ela-CreERT2;LSL-m*Shh*;m*Smo*. For the mice bearing tamoxifen-inducible ERT2 alleles, induction was carried out at the age of 6 weeks after birth by intraperitoneal injection of tamoxifen on five consecutive days as described previously [24]. Saline-injected mice of the same genotype were used as controls. Cohorts of at least five tamoxifen-induced, as well as at least two uninduced mice of each genotype were then killed at two monthly intervals, for the period ranging from 2 to 12 months after tamoxifen injection. At the end of the follow-up period, mice were killed by CO_2 insufflation, and the pancreata were harvested and fixed in 10% neutral buffered formalin solution for paraffin embedding. In
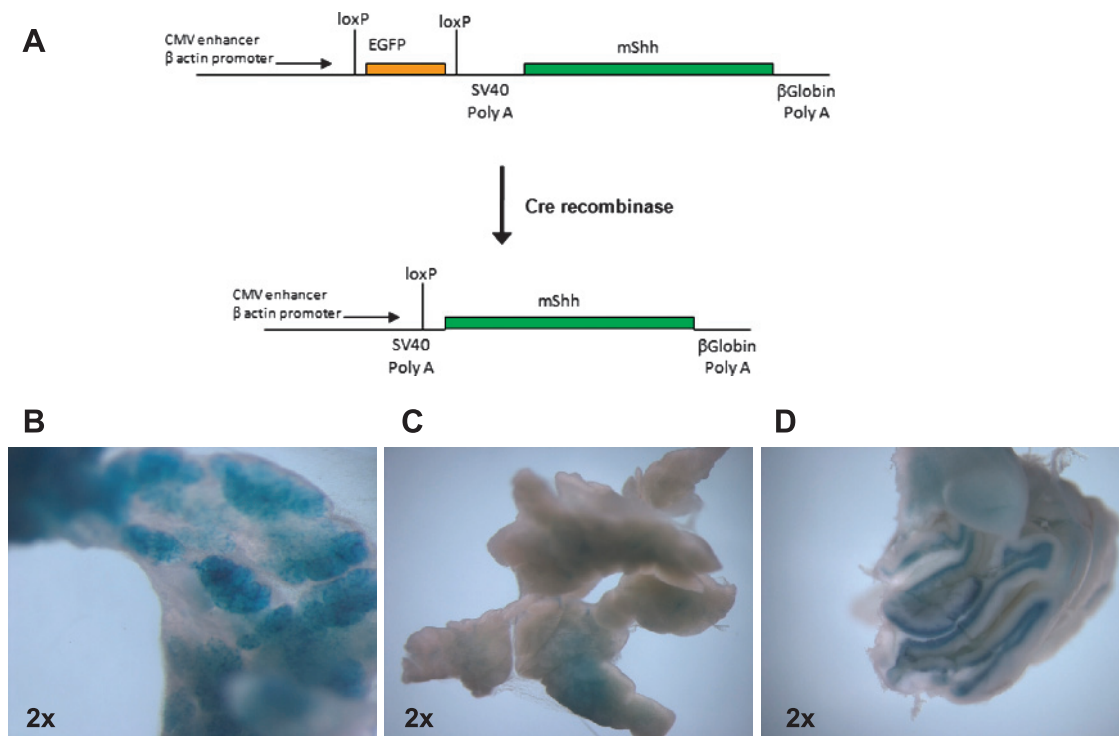
Figure 1. Targeting endogenous Shh expression to the adult murine pancreas. (A) The presence of the transgenic construct in somatic cells can be verified by enhanced green fluorescent protein expression. *Cre*-mediated recombination of the LSL cassette leads to excision of the enhanced green fluorescent protein cassette and expression of Shh (described previously in Hingorani et al. [17]). (B) Positive X-Gal staining revealed robust Cre-mediated recombination in the exocrine pancreas of *Ela*-CreERT2;LSL-m*Shh*;*Rosa*26R reporter mice after induction with tamoxifen. (C) Uninduced mouse. (D) Cerebellum of heterozygous *Ptch-lacZ* mice served as a positive control for X-Gal staining.

some cases, parts of the pancreata were immediately snap frozen in liquid nitrogen and then stored at −80°C until further use.

Immunohistochemistry

Immunolabeling of Shh and X-Gal staining were performed as previously described [24,26].

Immunofluorescence and Confocal Microscopy

Paraffin sections from Ela-CreERT2;LSL-m*Shh* murine pancreata were collected and treated with antigen retrieval solution (Dako, Hamburg, Germany). The sections were probed overnight with the following antibodies: rabbit anti-insulin (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti–Pan-cytokeratin (Abcam, Cambridge, United Kingdom), rabbit anti-amylase (Abcam), and mouse anti–acetylated-tubulin (Sigma, St Louis, MO). After washes in 0.1% PBS–Triton X-100, fluorescent detection was performed using Alexa Fluor 488 and 546 (Invitrogen, Carlsbad, CA)–conjugated secondary antibodies. Images were acquired using a Zeiss LSM 510 confocal microscope and digitized using Zeiss Image Browser (Zeiss, Jena, Germany).

Results

Developmental Overexpression of Shh Ligand in the Pancreatic Anlage Results in Malformation and Perinatal Lethality

In line with previous reports by others using comparable systems (Pdx1-Shh transgenic mice) [6], developmental overexpression of Shh led to pancreatic malformation and embryonic lethality in *Pdx1*-Cre;

LSL-m*Shh* mice (Figure W1). The pancreata were characterized by mucinous metaplastic glands and an expansion of intestinal-type mesenchyme, analogous to what has been described with transgenic ligand expression. These experiments confirmed the *in vivo* recombinatorial efficacy of the LSL cassette and the ability to generate an ectopic Shh ligand.

Aberrant Hh Ligand Expression in the Mature Pancreas Leads to Mesenchymal Expansion without Intraductal Lesions

In light of the developmental anomalies observed with *Pdx1*-Cre; LSL-m*Shh* mice, we generated cohorts of *Ela*-CreERT2;LSL-m*Shh* mice capable of producing Hh ligands in the adult exocrine pancreas. The *Ela*-CreERT2 allele is expressed within the mature acinar compartment on tamoxifen induction, as described [23]. To assess the efficiency of *Cre*-mediated recombination within the adult exocrine pancreas, we first generated tamoxifen-induced *Ela*-CreERT2;*Rosa*26R reporter mice, which confirmed the robustness of the *Elastase* promoter in conditional removal of the LSL cassette (Figure 1*B*); cerebellar beta-galactosidase expression in heterozygous *Ptch-lacZ* mice was used as positive control for the X-Gal staining (Figure 1*C*). In contrast to the *Pdx1*-Cre;LSL-m*Shh* mice, embryonic malformations or subsequent growth defects were not observed in the *Ela*-CreERT2;LSL-m*Shh* mice.

Cohorts of *Ela*-CreERT2;LSL-m*Shh* mice were then observed for up to 1 year after tamoxifen induction, with at least five induced and two uninduced mice being killed at each interval of 2 months, beginning at the second month after tamoxifen injection. As expected, the uninduced mice did not demonstrate any microscopic abnormalities in

the pancreas at any period during follow-up (Figure 2*A*). In contrast, tamoxifen-induced mice demonstrated a progressive mesenchymal expansion within the periacinar stroma, with the earliest histologic evidence for the spindle-shaped cells seen as early as 2 months after induction (Figure 2, *B-D*). Unlike the mutant *Kras*-driven mouse models of pancreatic neoplasia, aberrant expression of Shh ligand alone did not result in exocrine adenocarcinomas, intraductal precursor lesions, or even widespread acinar-ductal metaplasia, including that in mouse pancreata examined at 1 year after induction.

To address the possibility that the observed lack of Hh ligand responsiveness of the epithelial compartment was due to absence of the Smoothened (Smo) receptor, the entire study was repeated with acinar cell coexpression of m*Smo* along with m*Shh* by generating *Ela*-CreERT2;LSL-m*Shh*;LSL-m*Smo* compound transgenic mice. The ani-

mals were induced in the same manner as outlined above and observed for up to 12 months after tamoxifen induction. Notably, the pancreatic phenotype observed in the *Ela*-CreERT2;LSL-m*Shh*;LSL-m*Smo* mice was identical to that in the *Ela*-CreERT2;LSL-m*Shh* mice, that is, no intraductal lesions or neoplasms were identified, whereas a marked expansion of periacinar stromal cell compartment was found, as already described above (Figure 2, *E-F*). These results ruled out the possibility that the observed lack of Hh responsiveness of acinar cells was simply due to an overall lack of Smo expression as hypothesized originally.

Responsiveness to Ectopic Hh Ligand Is Restricted to the Stromal Compartment

To further characterize the dynamics of Hh ligand–dependent signaling in the exocrine pancreas, we assessed the localization of Shh,
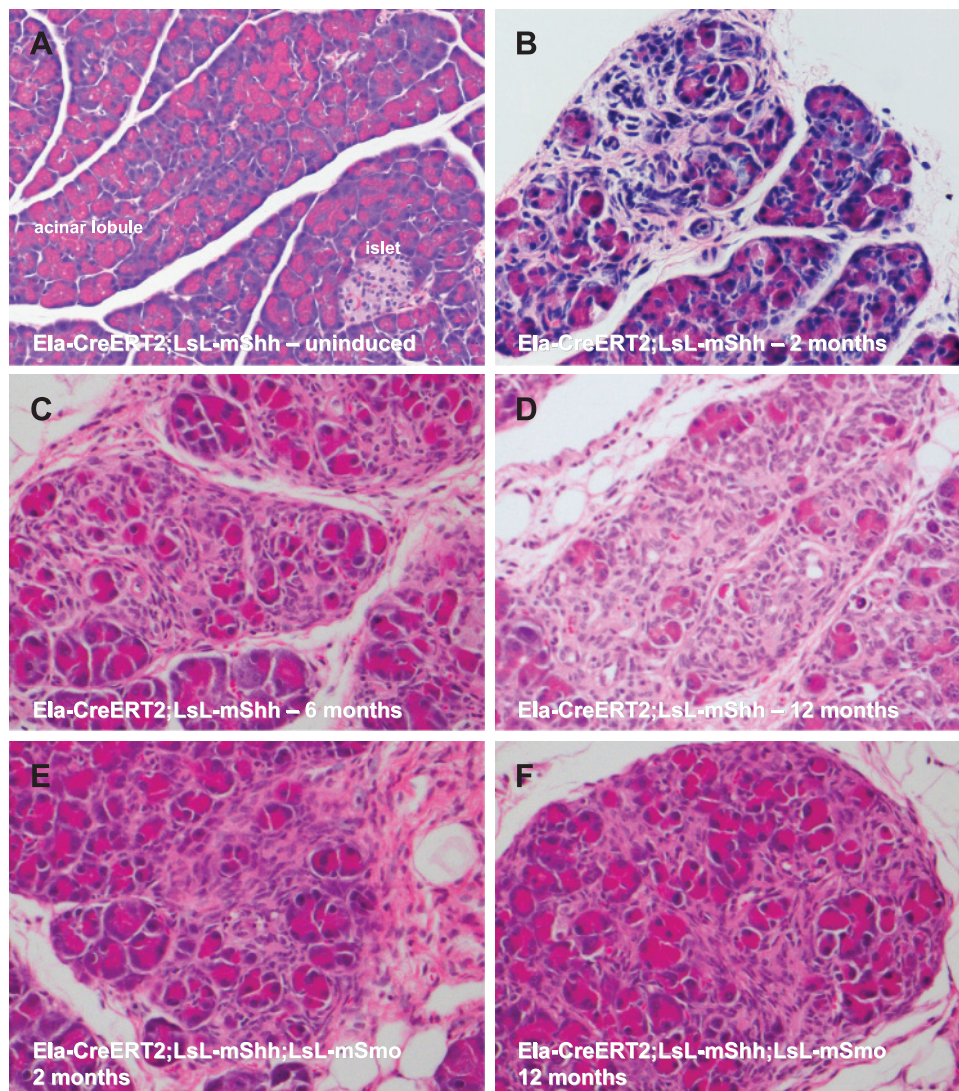
Figure 2. Hh misexpression in the adult murine pancreas leads to mesenchymal expansion in the absence of intraductal lesions. (A) No morphologic abnormalities were observed in the pancreata of *Ela*-CreERT2;LSL-m*Shh* mice in the absence of tamoxifen induction. (B-D) After tamoxifen induction, morphologic changes in the *Ela*-CreERT2;LSL-m*Shh* pancreata were observed as early as 2 months and persisted at 1 year of follow-up, at which time the study was terminated. The most conspicuous alteration was a marked expansion of stromal cells between existing acinar lobules, displacing the acinar cells over time. Photomicrographs were obtained at 2 (B), 6 (C), and 12 months (D) after tamoxifen induction, respectively. In contrast, no epithelial alterations such as intraductal precursor lesions or exocrine cancers were found. (E and F) Comparable histologic alterations were observed in tamoxifen-induced *Ela*-CreERT2;LSL-m*Shh*; LSL-m*Smo* mice, beginning as early as 2 months after induction (E) and persisting up to 1 year (F). No intraductal precursor lesions or exocrine cancers were seen in these cohorts either.
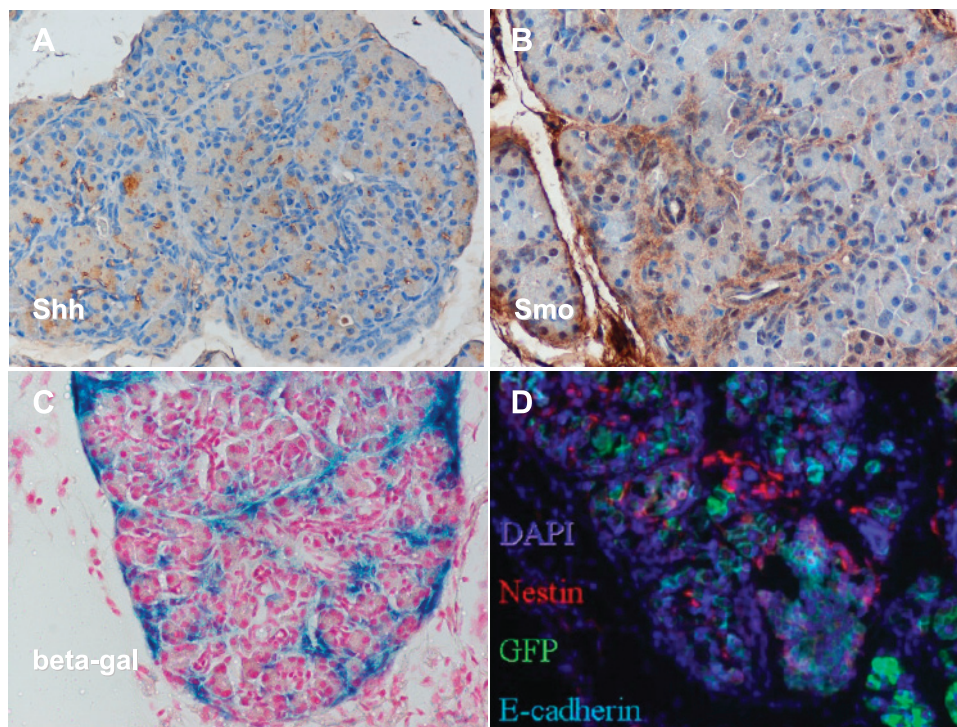
Figure 3. Confirmation of stromal Hh pathway activity on *Cre*-mediated recombination in the exocrine pancreas. (A) Immunohisto-chemistry confirmed the expression of Shh ligand in most acinar cells in tamoxifen-induced *Ela*-CreERT2;LSL-m*Shh* mice. (B) In contrast, Smo expression was essentially restricted to the expanded periacinar stromal compartment in tamoxifen-induced *Ela*-CreERT2; LSL-m*Shh* mice. (C) The expanded stromal compartment had evidence of Hh activation, as confirmed by expression of beta-galactosidase (blue) in tamoxifen-induced *Ela*-CreERT2;LSL-m*Shh*;*Ptch-lac*Z reporter mice. This pattern overlapped with that of Smo receptor expression in B. (D) The stromal population was negative for E-cadherin and expressed nestin, a feature of mesenchymal cells in the adult pancreas.

Smo, and the Hh gene target *Ptch* in tamoxifen-induced mice. Over-expression of Shh was observed in most acinar cells within the murine pancreata by immunohistochemistry (Figure 3*A*), whereas immuno-labeling of Smo was essentially confined to adjacent stromal cells (Figure 3*B*). The localization of *Ptch*, assessed using *Ela*-CreERT2; LSL-m*Shh*;*Ptch-lac*Z reporter mice, mirrored that of Smo protein, confirming that the stromal compartment was the principal recipient of the epithelial Hh ligand (Figure 3*C*). We were unable to demonstrate any convincing acinar-specific beta-galactosidase expression in the examined pancreata. In line with a mesenchymal nature of the *Ptch*-expressing periacinar stromal compartment, immunofluorescence dem-onstrated robust expression of nestin, a marker of mesenchymal cells in the adult pancreas [27], whereas the epithelial antigen E-cadherin was absent (Figure 3*D*).

Primary Cilia Are Absent on Cells within the Acinar Compartment

Recent evidence suggests that the presence of primary cilia and re-cruitment of the receptor protein Smo to the cilia are prerequisites for the ability of a cell to respond to stimulation by Hh ligands, irrespec-tive of the presence of other pathway components [28]. Therefore, the presence of primary cilia was analyzed in pancreatic cryosections using immunofluorescence for acetylated tubulin, a marker for primary cilia [29]. Acetylated tubulin expression was observed within the ductal epithelium, on pancreatic endocrine cells within islets of Langerhans, and the expanded periacinar stromal compartment, but not in acinar cells of *Ela*-CreERT2;LSL-m*Shh* mice (Figure 4). The absence of pri-

mary cilia in acinar cells likely explains the lack of demonstrable cell autonomous effects from the secreted Hh ligand, including in com-pound transgenic *Ela*-CreERT2;LSL-m*Shh*;LSL-m*Smo* mice.

Discussion

The identification of aberrant Hedgehog signaling in pancreatic can-cer has led to significant research efforts aimed at exploiting this pathway for the development of novel therapeutic options during the past few years [5,6,30]. In fact, at present, there are several Hedgehog small-molecule inhibitors available that are undergoing initial clinical eval-uation and have shown promising *in vivo* efficacy in various human cancers, including medulloblastoma and basal cell carcinoma [13,31–35].

However, despite this obviously encouraging progress in moving for-ward the translational component, there is an incomplete understand-ing of the mechanisms underlying the effects of aberrant Hh signaling in pancreatic cancer. Specifically, there have been conflicting reports as to the nature of cells within ductal adenocarcinomas that are competent to receive, and respond to, secreted Hh ligands [15,16]. The initial studies suggested that ligand expression and activation of the Hh path-way occurred within the "bulk" population of neoplastic epithelial cells in a cell autonomous manner [5,6]. Subsequent studies proposed a somewhat restricted subpopulation of Hh-dependent neoplastic cells within the epithelial compartment, specifically those with tumor-initiating and metastatic dissemination capacity [7,9,11,36,37].

The most recent series of studies in pancreatic adenocarcinoma sug-gest that the ability to respond to paracrine Hh ligand rests predomi-nantly within stromal cells rather than the neoplastic cells themselves

[38,39]. Bailey et al. [40] demonstrated that peritumoral desmoplasia, a feature most pronounced in pancreatic adenocarcinomas, is a consequence of the paracrine activity of epithelial Hh ligand on stromal cells. Furthermore, Olive et al. [10] showed that Hh inhibition using a small-molecule inhibitor led to marked stromal depletion and en-hanced drug delivery in an autochthonous mouse model of pancreatic cancer. Similarly, Lauth et al. recently showed marked overexpression of the Hh target gene *Gli1* in tumor-associated stromal cells, but only minimal expression in pancreatic adenocarcinoma, consistent with a high pathway activity in the former but not the latter compartment
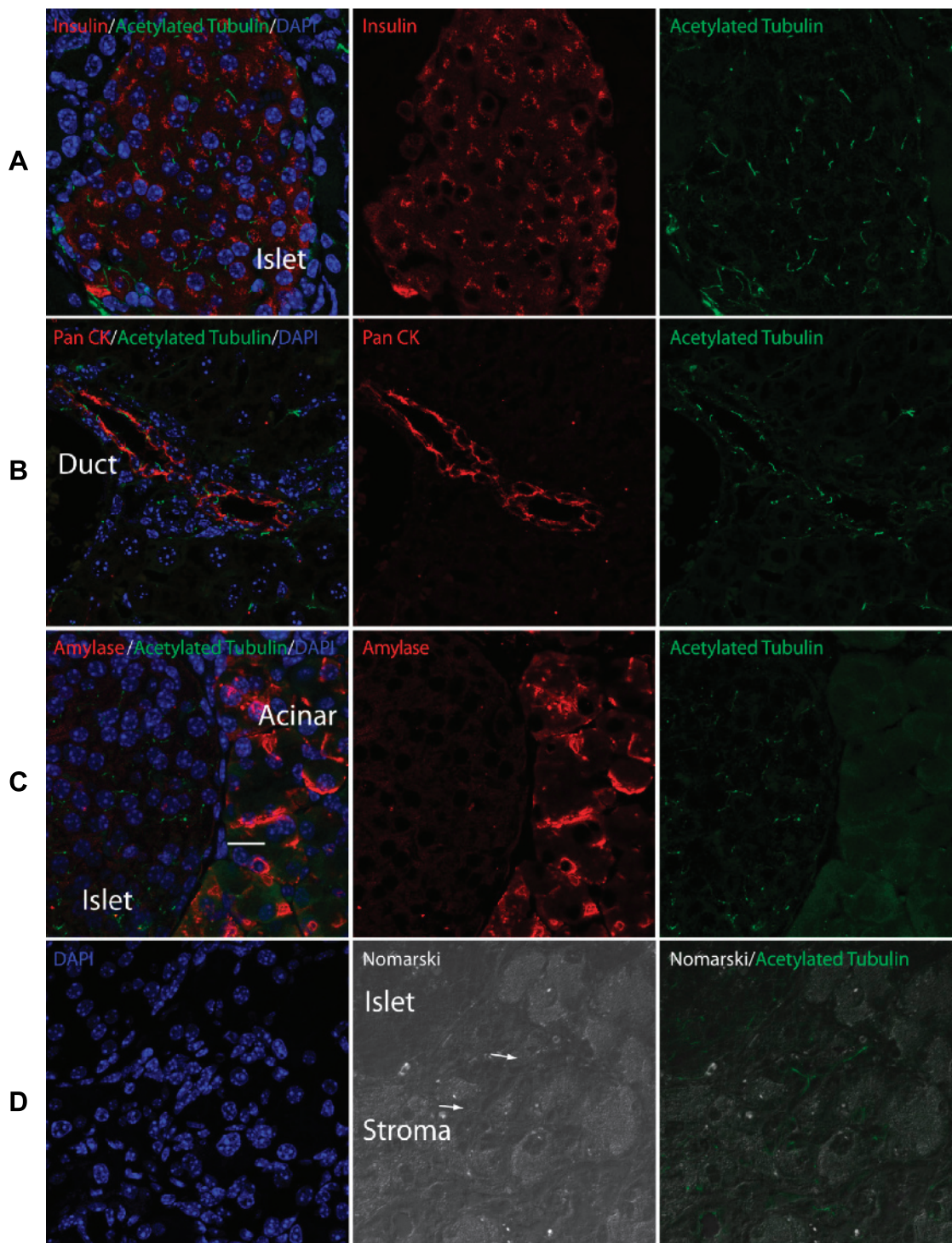
Figure 4. The expanded stromal compartment expresses markers of primary cilia. Acetylated tubulin expression was observed in (A) islets marked by insulin and in (B) ducts marked by pan-cytokeratin (Pan CK) from *Ela*-CreERT2; LSL-m*Shh* murine pancreata. In contrast, acetylated tubulin could not be detected by staining in amylase expressing acinar cells (C). (D) Stromal cells (white arrows) intermixed within exocrine structures were identified using Nomarski optics. Expression of acetylated tubulin in these cells was consistent with the presence of primary cilia within the stroma. Staining for insulin, pan CK, acetylated tubulin, and amylase as indicated. Scale bar, 10 μm.

[41]. The dispensable nature of Hh signaling within the epithelial compartment is reiterated by recent studies, which confirmed that biallelic inactivation of *Smo* in pancreatic epithelial cells did not affect subsequent development of adenocarcinoma in an autochthonous model [42], whereas conversely, *Smo* inactivation in stromal cells led to delayed growth of pancreatic cancer xenografts [38].

Prior reports examining the role of aberrant Hh signaling in the epithelial compartment have largely relied on expression of a constitutively active m*Gli2* allele that lacks the N-terminal repressor domain (GLI2ΔN) [20]. Thus, *Pdx1*-Cre;*CLEG2* mice conditionally overexpressing GLI2ΔN in the pancreas develop poorly differentiated cancers in the absence of concomitant mPanIN lesions. Notably, the use of a constitutively active m*Gli2* allele bypasses Hh ligand dependence, which is most relevant to the cognate pathophysiology in the human pancreas. Similarly, a study by Thayer et al. [43] reported on mice expressing a constitutively active form of Smoothened (R26-SmoM2) under the control of a ubiquitously expressed inducible Cre transgene (CAGGS-CreER). Interestingly, on tamoxifen induction, a high rate of novel cystic metaplastic lesions was observed in the pancreata of CAGGS-CreER;R26-SmoM2 mice, largely replacing the normal pancreatic architecture and showing histomorphologic features reminiscent of mucinous cystic neoplasms.

To the best of our knowledge, ours is the first study that examines ligand-dependent Hh signaling in the adult murine exocrine pancreas, with or without additional coexpression of an m*Smo* allele. We demonstrate that expression of Shh ligand in the adult exocrine pancreas is, by itself, not sufficient to cause the formation of either noninvasive intraductal mPanIN lesions or invasive adenocarcinomas. On the contrary, and not unexpectedly, our cohorts of mice demonstrate a marked expansion of a periacinar stromal cell compartment, reiterating that, in the absence of concomitant oncogene expression (e.g., *Kras*) or exocrine injury (i.e., pancreatitis), stromal cells remain the major Hh ligand recipients in the adult pancreas. Using *Ptch-lacZ* reporter mice, we observe X-Gal staining that is restricted to the expanded periacinar stromal cells, confirming their ability to respond to exogenous Hh signals.

Multiple lines of evidence have now demonstrated that the presence of primary cilia are a prerequisite for Hh signal transduction [44,45]. On binding of Hh ligand to the membrane receptor Ptch, the inhibition on Smo is released and Smo is recruited to the cilium [28,46]. Furthermore, SuFu represents a major negative regulator of Hh signal transduction in vertebrate cells [47–50], and ligand-driven activation of the Hh pathway might lead to subcellular translocation of a protein complex containing SuFu and Gli to the tip of primary cilia, where the inhibitory function of SuFu on Gli is released, so that the latter can shuttle to the nucleus and induce transcription of Hh target genes, thus exemplifying the pivotal role of primary cilia in this process [51]. Notably, we find expression of acetylated tubulin, a primary cilia marker, in islet cells, the ductal epithelium, and in the expanded periacinar stromal cells of the adult pancreas but none within acinar cells. The absence of primary cilia likely explains the inability of mature acinar cells to respond to secreted Hh ligand in a cell autonomous manner, even in the setting of enforced coexpression of the Smo receptor. A prior report by Seeley et al. [52] confirms that murine ductal epithelium and islets do harbor expression of ciliary markers, consistent with our own data. The lack of demonstrable pathology within islets or the ductal epithelium is somewhat surprising, given their apparent competence to respond to Hh signals and suggests that these compartments have other checkpoints in place, especially in the absence of additional oncogenic "driver" influences.

In conclusion, in this study, we describe a genetically engineered mouse model of Hh pathway activation through Shh ligand overexpression in acinar cells of the adult pancreas. Shh overexpression led to a striking expansion of a periacinar stromal cell compartment but did not induce formation of premalignant precursor lesions or overt pancreatic neoplasia, in line with stromal cells being the major Hh-responsive elements in the mature organ. This autochthonous model serves as a platform for studying epithelial stromal interactions in pancreatic carcinogenesis, as well as the role of cooperating genetic alterations with aberrant Hh signaling.

Acknowledgments

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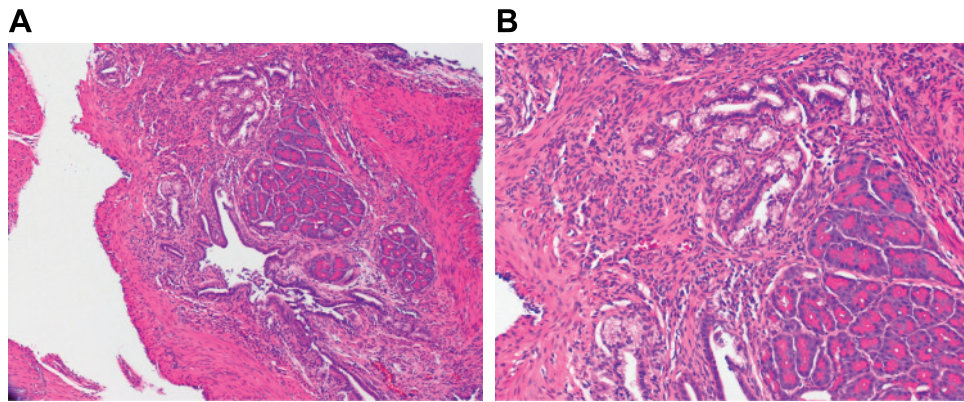
Figure W1. Developmental overexpression of Shh ligand leads to pancreatic malformation. In *Pdx1*-Cre;LSL-m*Shh* mice, uniform perinatal lethality was observed. Necropsy confirmed the presence of a malformed pancreas with marked mesenchymal expansion and mucinous metaplasia of remnant ducts. Hematoxylin and eosin stains; A and B are ×10 and ×20 images from a single pancreas.