Hedgehog Signaling in Pancreas Epithelium Regulates Embryonic Organ Formation and Adult β-Cell Function

Janet Lau and Matthias Hebrok

OBJECTIVE—Current studies indicate that Hedgehog (Hh) signaling must be excluded during early stages of pancreas formation. However, conflicting evidence suggests that Hh signaling may be active later during pancreas formation and that it is required for insulin production and secretion in cultured β-cell lines. The objective of this study was to address these discrepancies by assessing the in vivo role of epithelial Hh signaling in the pancreas.

RESEARCH DESIGN AND METHODS—To identify Hh-active cells in the developing and adult pancreas epithelium, we characterized transgenic reporter Patched1-LacZ mice. To determine the requirement for epithelial Hh signaling in the pancreas, we eliminated an essential Hh signaling component, Smoothened (Smo), in the pancreatic epithelium, and assessed pancreatic development and adult β-cell physiology phenotypes.

RESULTS—Characterization of Patched1-LacZ reporter mice revealed low-level LacZ expression in pancreatic epithelial cells throughout development until birth, when LacZ activity increases in intensity specifically in endocrine and ductal cells. In the absence of Hh signaling, Smo-deficient mice have delayed pancreas formation leading to a temporary reduction in pancreatic epithelium and β-cell numbers. Although β-cell numbers recover by birth, adult Smo-deficient mice display glucose intolerance, increased insulin sensitivity, and reduced total insulin production.

CONCLUSIONS—These data show that Hh signaling functions early during pancreas morphogenesis to regulate epithelial and β-cell expansion and to modulate glucose metabolism by regulating insulin production in adult mice. *Diabetes* 59:1211–1221, 2010

The advancements in stem cell technologies carry the promise toward developing a renewable supply of β-cells and a cure for diabetes. While significant progress has been made in recent years, there is still a need for a greater understanding of the signals that influence pancreas and endocrine differentiation and function. To address this issue, we set out to define the role of the Hedgehog (Hh) signaling pathway in the pancreas epithelium and in β-cell formation and function.

Starting at about embryonic day 9.0 (e9.0) in mice, the pancreatic epithelium expands into the surrounding mesenchyme (1). The initial budding of the dorsal epithelium is followed by ventral epithelial budding 1 day later. Subsequently, the epithelial buds branch and differentiate into the exocrine acini and ducts, which produce and collect the digestive enzymes necessary for nutrient absorption, and the endocrine islets, which produce hormones essential for glucose utilization and storage. During this process, the pancreatic epithelium and mesenchyme compartments communicate with each other through the release of soluble signals that bind to their respective receptors expressed on target cells (2,3).

One pathway known to relay signals between epithelial and mesenchymal cells is the Hh signaling pathway, which is involved in morphogenesis and cell differentiation in many organs during embryogenesis. In mammals, three secreted ligands, Sonic Hh (Shh), Indian Hh (Ihh), and Desert Hh (Dhh), activate Hh signaling through Smoothened (Smo), a G-protein coupled receptor (GPCR)-like protein. Activation of Smo occurs when Hh ligands bind the receptor Patched1 (Ptc1), initiating activation of Hh target gene expression through the family of Gli transcription factors. Notably, some known Hh target genes include Hh signaling components themselves, including *Ptc1* and *Gli1*. While a number of other molecules have been characterized in the pathway, a more comprehensive explanation of their function is well described in several recent reviews (4–7).

Previous studies have demonstrated how Hh signaling affects pancreas formation. Characterization of *Hh* gene expression showed that *Shh* is expressed throughout the endoderm epithelium but is strikingly excluded from the specified pancreatic region, suggesting an inhibitory role of the protein in pancreas organogenesis (8). Indeed, this conclusion was supported in several studies (9–12) where models of activated Hh signaling resulted in loss of pancreatic tissue with a corresponding gain of tissues with duodenal properties. Interestingly, the varying level of Hh activation achieved in these models may correlate to varying differences in pancreas-to-duodenal conversion phenotypes, thus suggesting a dose dependent response to Hh activation. Complementary loss-of-Hh-activation studies (13–15) demonstrate that inactivation leads to an expansion of pancreatic area. Thus, these studies indicate that Hh signaling acts to limit pancreatic growth.

Despite the evidence that overt Hh signaling blocks pancreas organogenesis, several studies have demonstrated a positive role for Hh signaling in the pancreas. In the developing pancreas, *Ihh* and *Dhh* ligands and *Ptc1* receptor are expressed from e13.5 and onward in the developing pancreas and the adult islet and ductal tissues (13,16). Furthermore, studies by Thomas and colleagues in 2000 and 2001 (16,17) indicate that Hh signaling functionally supports insulin production and secretion by regulating *Pdx1* expression in INS1 cells. Thus, these findings suggest a bimodal role for Hh signaling, with low-level
signaling required for some aspects of development and endocrine function while overt activation inhibits organogenesis. However, previous studies failed to address the cellular requirements for Hh signaling in the epithelial versus the mesenchymal compartments. As Hh signaling has been shown to differentially modulate epithelial and mesenchymal development in other contexts, a closer examination of the role of Hh signaling in either compartment is needed.

In this study, we investigated the effects of loss of Hh signaling in the epithelium during development and in the postnatal islet. By employing Ptc1-LacZ transgenic mice, a reporter line that marks cells with active Hh signaling, we first demonstrate that Hh is active within the pancreatic epithelium. Second, to address the functional role of epithelial Hh signaling, we used Pdx1-Creearly mice to generate transgenic mice lacking Smo function specifically in the pancreas epithelium. Results from these studies show that epithelial-specific Smo loss results in delayed expansion of the early pancreatic epithelium and delayed β-cell morphogenesis. While β-cell numbers recover by birth, Smo mutant mice are glucose intolerant and produce less insulin despite increased β-cell mass. Thus, our studies demonstrate a requirement for epithelial Hh signaling both during formation of the embryonic pancreas epithelium as well as in the maintenance of endocrine function.

**RESULTS**

**Hh-active cells exist within the developing and adult pancreas.** Despite intensive research, the absence of reliable antibodies to detect signaling components in tissue sections has left the characterization of Hh-active cells within the pancreas unclear. To determine whether pancreatic cells display active Hh signaling, we stained developing and adult pancreata from Ptc1-LacZ (Ptc1-LacZ) transgenic mice. In these mice, the bacterial b-galactosidase (b-gal) gene has replaced the endogenous Ptc1 coding sequence, and its activity is controlled by the regulatory elements of the Ptc1 locus (20). Since Ptc1 itself is a transcriptional target gene of Hh signaling, b-gal activity marks Hh active cells. We found b-gal–positive cells in the pancreatic epithelium as early as e10.5 (Fig. 1A–D, arrowheads). Notably, we also detected a few cells in the adjacent mesenchyme with punctate b-gal staining (Fig. 1C and D, arrows). Between e12.5 and e17.5, we observed a progressive increase in b-gal–positive cells (Fig. 1E–F), with cells confined to the endocrine and ductal epithelium after birth (Fig. 1Q–Z). Remarkably, postnatal b-gal–positive cells were marked by a qualitatively more robust signal throughout the cell. Thus, our results demonstrate that Hh-active cells reside in the early pancreatic epithelium, as well as in the adult endocrine and ductal lineage.

**Loss of Hh signaling in Pdx1-Creearly;Smofoxnull pancreas.** Smo, an essential mediator of Hh signaling, initiates Gli transcriptional activity in all Hh-active cells. Tissue-specific elimination of Smo function has been shown to block ligand-induced Hh signaling (21–24). Using the same strategy, we decided to use Pdx1-Creearly mice to eliminate the floxed allele of Smo in the pancreatic epithelium. Earlier work by our group has shown that Pdx1-Creearly mice induce efficient Cre-mediated recombination throughout the pancreatic epithelium by e10.5 (25). To confirm that Pdx1-Creearly;Smofoxnull pancreata had efficiently recombinated the floxed Smo allele, we stained pancreata for Smo protein expression. Similar to Ptc1-LacZ expression, Smo staining in Smofoxwildtype or Smofoxnull control tissues was undetectable by immunostaining in the exocrine pancreas (not shown) but present in insulin-expressing cells at P0 (Fig. 2A, C, and E). In contrast, Smo protein was efficiently eliminated in Pdx1-Creearly;Smofoxnull mutants (Fig. 2B, D, and F), a finding supported by quantitative PCR that detected a 70% downregulation of Smo gene expression in adult islets (Fig. 2G) and e12.5 whole pancreatic buds (data not shown). Complete elimination of Smo expression by quantitative PCR is not expected as the Cre line does not induce recombination in the surrounding mesenchyme or other nonpancreatic cell types (e.g., endothelial and neural cells) within the isolated pancreatic islet or tissue. To verify that elimination of Smo resulted in downregulation of Hh signaling, we performed quantitative PCR to analyze the expression of Hh target genes, Gli1 and Pich1, and found their expression dramatically and significantly downregulated in Pdx1-Creearly;Smofoxnull adult islets (Fig. 2H and I). Thus, we conclude that Pdx1-Creearly;Smofoxnull mice have strong reduction in Hh signaling in the pancreatic epithelium.
FIG. 1. Hedgehog-active cells reside in the developing and adult pancreas. Pancreatic tissue was isolated from either control mice at e10.5 (A), e12.5 (E), e15.5 (I), e17.5 (M), postnatal day 0 (P0) (Q), and 2 weeks (U), or Patched1-LacZ (Ptch1-LacZ) transgenic mice at e10.5 (B–D), e12.5 (F–H), e15.5 (J–L), e17.5 (N–P), P0 (R–T), 2 weeks (V–X), and 4 weeks (Y–Z’). β-Gal activity was marked by blue staining pattern highlighted by arrowheads and/or arrows. Arrowheads = Ptch1-LacZ staining in the epithelium; arrows = Ptch1-LacZ staining in the mesenchyme. Scale bars in A and B, E and F, I and J, and M and N are equal to 50 μm, while scale bars in Q and R, and U and V, are equal to 100 μm. Lastly, the scale bar in Y is equal to 200 μm. (A high-quality digital representation of this figure is available in the online issue.)
**FIG. 2.** *Pdx1-Cre<sup>early</sup>;*Smoflox/null* mice have downregulated Smo expression and Hh signaling. Smoothened (green) and insulin (red) costaining in control (A, C, and E) and *Pdx1-Cre<sup>early</sup>;*Smoflox/null (B, D, and F) P0 islets. Scale bars are equal to 10 μm. G: Smoothened gene expression by Sybr green real-time PCR is downregulated in islets isolated from *Pdx1-Cre<sup>early</sup>;*Smoflox/null mice vs. control islets (*n* = 3; ***P* < 0.005). Expression of Hh target genes Gli1 (H) and Patched1 (I) is reduced compared with controls (*n* = 4; ***P* < 0.005). *P* values were determined by Student *t* test. (A high-quality digital representation of this figure is available in the online issue.)

**Pdx1-Cre<sup>early</sup>;*Smo<sup>flox/null</sup>** pancreata show disrupted pancreas morphogenesis. Previous loss-of-function studies suggested that widespread reduction of Hh signaling in the epithelium and mesenchyme results in an expansion of the pancreatic domain and a relative increase in endocrine area (13–15). To determine whether downregulated Hh signaling in the pancreatic epithelium alone had similar effects, we analyzed the pancreatic epithelium during early pancreas formation. Control and mutant *Pdx1-Cre<sup>early</sup>;*Smoflox/null embryos were harvested at e12.5, and whole pancreata were stained with anti-Pdx1 and anti-Nkx6.1 antibodies to mark pancreatic epithelial cells. Staining for Pdx1 and Nkx6.1 revealed that cellular differentiation was unaffected as all epithelial cells were positive for both markers (Fig. 3A and B). Next, we assessed pancreas morphology and size by histological analysis of whole pancreata. In contrast to previous reports that correlate inhibition of overall Hh signaling with increased pancreas size, we found that the pancreatic epithelial area was decreased by nearly 60% at e12.5 in *Pdx1-Cre<sup>early</sup>;*Smoflox/null mutants (Fig. 3C). Furthermore, the epithelium appeared more disorganized, suggesting that epithelial branching was disrupted (Fig. 3A). Consecutive serial sections of e12.5 pancreatic epithelium more clearly demonstrated that epithelial expansion and branching were disrupted (supplemental Fig. 1A–F). The disruption in expansion and branching is temporary, as by e15.5 total pancreatic epithelial area normalized (Fig. 3C) and showed no gross abnormalities at birth. Recovery of total pancreatic epithelial area by e15.5 may be due to changes in cell proliferation or cell death. To determine whether these processes were altered at this stage, we stained e15.5 tissues with either phospho-histone H3 to mark proliferating cells or for cleaved caspase 3 to mark apo-
ptotic cells. Quantitative analysis showed increased number of proliferating epithelial cells in Pdx1-Creearly; Smo<sup>flox/null</sup> mutant pancreata (Fig. 3D), while the number of apoptotic cells was unchanged (Fig. 3E). Thus, recovery of total pancreatic epithelial area by e15.5 is due to increased epithelial proliferation.

To address the mechanism of early epithelial loss in Pdx1-Cre<sup>early</sup>; Smo<sup>flox/null</sup> pancreata, we examined the effect decreased Hh signaling had on other developmental signaling pathways. From e10 to e11.5, Fgf10 is secreted from the mesenchyme and promotes expansion of pancreatic progenitor populations (26 –29). Although previous work from our lab showed that increased Hh signaling in embryos mutant for the Hh inhibitor Hhip (<sup>Hhip<sup>−/−</sup></sup>) reduces Fgf10 expression in the mesenchyme, and impairs pancreatic epithelial growth and branching (10), it is unclear whether changes specifically in epithelial Hh signaling secondarily impact mesenchyme function and Fgf10 expression. To determine whether epithelial Hh signaling is required for mesenchymal Fgf10 expression, we performed quantitative PCR on mesenchyme-intact e10.5 and e11.5 Pdx1-Cre<sup>early</sup>; Smo<sup>flox/null</sup> pancreata but detected no changes in expression level (supplemental Fig. 2A). Besides fibroblast growth factor (FGF) signaling, Wnt and

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**FIG. 3.** Early pancreas formation in Pdx1-Cre<sup>early</sup>; Smo<sup>flox/null</sup> mice is perturbed. Sections stained with Pdx1 (green), Nkx6.1 (red), and DAPI (blue) staining in e12.5 pancreata show disrupted pancreatic branching in control (A) vs. Pdx1-Cre<sup>early</sup>; Smo<sup>flox/null</sup> (B) mice. Scale bars are equal to 100 μm. (Arrowheads in A and B indicate branching tips.) C: Total pancreatic epithelial area is reduced in Pdx1-Cre<sup>early</sup>; Smo<sup>flox/null</sup> mice at e12.5 but normalized by e15.5. (n = 4 for e12.5 samples; **P < 0.02; n = 6 for e15.5 samples). D: Recovery of total pancreatic epithelial area at e15.5 is due to increased total epithelial cell proliferation, as measured by phospho-histone H3 (n = 5; ***P < 0.005). E: The apoptotic rate in e15.5 total pancreatic epithelium is not changed in Pdx1-Cre<sup>early</sup>; Smo<sup>flox/null</sup> mice (n = 3). P values were determined by Student t test. (A high-quality digital representation of this figure is available in the online issue.)
bone morphogenetic protein (BMP) signaling pathways also interact with Hh signaling to regulate organ formation and differentiation (25,30–36). In particular, we have shown previously that stabilization of β-catenin early during organogenesis results in a severe loss of pancreatic epithelium and increased epithelial expression of Hh ligands (25). To determine whether cell autonomous loss of Hh signaling affected expression of Wnt, BMP, or Hh ligands at this stage, we performed quantitative PCR for soluble factors suggested that Wnt signaling might be increased at e10.5, expression of Wnt target genes was not altered (supplemental Fig. 2C and D). In addition, we did not detect significant changes in gene expression levels for BMP or Hh ligands (supplemental Fig. 2B and E). Therefore, we conclude that reduction in Hh signaling in the epithelium results in delayed epithelial expansion, while mesenchymal signaling appears unchanged during early pancreatogenesis. 

Pdx1-Creearly;Smoflox/null pancreata show disrupted β-cell morphogenesis. Next, we assessed the effects of downregulated Hh signaling on endocrine and β-cell development. Grossly, exocrine, duct, and endocrine differentiation appeared normal. However, upon closer examination of endocrine areas, we found that β-cell area was diminished at e15.5. Quantification of α-cell and β-cell areas revealed that while the relative α-cell area was normal, the relative β-cell area was reduced by 45% at e15.5 (Fig. 4A, B, and E). Interestingly, the decrease in β-cell area recovered to normal levels by birth (Fig. 4F). We hypothesized that increased β-cell proliferation and/or neogenesis could account for the recovery in β-cell area.

FIG. 4. Pdx1-Creearly;Smoflox/null mice have delayed β-cell formation. Insulin (green) and glucagon (red) staining in e15.5 pancreata show an approximate equivalent number of α-cells (140 α-cells in control vs. 130 α-cells in mutant samples) but a reduced number of β-cells (144 β-cells in control vs. 95 β-cells in mutant samples) between control (A) and Pdx1-Creearly;Smoflox/null (B) mice. Neurogenin3 (Ngn3) (green) and Nkx6.1 (red) staining in e15.5 control (C) and Pdx1-Creearly;Smoflox/null (D) pancreata. Scale bars in A–D are equal to 100 μm. Quantification of endocrine areas at e15.5 and P0 show that while glucagon areas are normal, insulin area is reduced at e15.5 (E) but normalizes by P0 (F) in Pdx1-Creearly;Smoflox/null mice (n = 3 for e15.5 samples; **P < 0.02; n = 4 for P0 samples). G: Proliferation measured by phospho-histone H3 does not account for β-cell recovery at e15.5 or P0 (n = 4; *P < 0.05). While analysis of Ngn3 (green)/Nkx6.1 (red) positive cells in control (C) and Pdx1-Creearly;Smoflox/null (D) mice show no significant change in the number of total Ngn3 positive cells (H), a 71% increase in the number of Ngn3/Nkx6.1 positive progenitor β-cells (I) is observed at e15.5 (n = 6; **P < 0.02). P values were determined by Student t test. (A high-quality digital representation of this figure is available in the online issue.)
As previously described, we used phospho-histone H3 to analyze cell proliferation. We found that the number of proliferating insulin cells was reduced at e15.5 but normal at P0 (Fig. 4G). Therefore, early increased proliferation of β-cells could not account for the documented recovery at P0 but may contribute to reduced β-cell numbers at e15.5.

To determine whether β-cell neogenesis was affected, we examined neurogenin 3 (Ngn3) expression in control versus Pdx1-Cre<sup>early</sup>;Smoflox/null mice. Ngn3 is a transcription factor expressed during endocrine cell fate specification, and cells that express Ngn3 and Nkx6.1 are a defined population of immature β-cell progenitors. While the total number of Ngn3-expressing cells was unchanged (Fig. 4H), we found that the number of immature Ngn3/Nkx6.1 double-positive β-cell progenitors were increased by 71% in Pdx1-Cre<sup>early</sup>;Smoflox/null mice at e15.5 (Fig. 4I). Together, these results indicate that a higher proportion of β-cell progenitors remain midway through pancreas development, suggesting that β-cell formation is either prolonged or delayed. To differentiate between these two processes, we characterized the gene expression pattern of Ngn3 from e12.5 through e16.5 by quantitative PCR. Although we did not find significant changes in Ngn3 expression levels (supplemental Fig. 3), we observed a trend toward a delayed peak in Ngn3 expression from e13.5 to e14.5 in Pdx1-Cre<sup>early</sup>;Smoflox/null mice. Moreover, Ngn3 expression at e16.5 is significantly higher in Pdx1-Cre<sup>early</sup>;Smoflox/null than control mice, consistent with a delayed differentiation of β-cells (supplemental Fig. 3).

Summarily, our results show an early delay in pancreas growth and branching followed by a transient delay in β-cell formation upon inhibition of Hh signaling in Pdx1-Cre<sup>early</sup>;Smoflox/null mice.

**Adult Pdx1-Cre<sup>early</sup>;Smoflox/null pancreata have impaired β-cell function.** In vitro studies (16,17) have suggested that Hh signaling plays a role in insulin production and secretion in insulinoma-derived β-cell lines. To determine whether reduction of Hh signaling affects adult pancreas and β-cell function in vivo, we performed histological and physiological analyses in adult Pdx1-Cre<sup>early</sup>;Smoflox/null pancreata. Histological analysis did not reveal any morphological differences between control and Pdx1-Cre<sup>early</sup>;Smoflox/null pancreata by hematoxylin and eosin staining (Fig. 5A and B), and we observed normal expression of endocrine, exocrine, and ductal markers (Fig. 5C–J). Thus, adult pancreas and islet morphology is indistinguishable from wild-type tissue.

To determine whether loss of Hh signaling impacts β-cell physiology, we challenged Pdx1-Cre<sup>early</sup>;Smoflox/null mice to respond to a glucose load. Despite the absence of morphological defects, Pdx1-Cre<sup>early</sup>;Smoflox/null mice were glucose intolerant by 3 months of age (Fig. 6A), a defect that progressively worsened with age (data not shown). Glucose intolerance can be caused by defects in insulin resistance in peripheral tissues or defects in insulin production/secreton in β-cells. To determine whether Pdx1-Cre<sup>early</sup>;Smoflox/null mice are insulin resistant, mice were challenged by an intraperitoneal injection of insulin and serum glucose levels were measured. Our results showed that in comparison to control mice, blood glucose levels in Pdx1-Cre<sup>early</sup>;Smoflox/null mice were significantly lower and took longer to recover (Fig. 6B), indicating that they do not have peripheral tissue defects in sensing insulin and were, in fact, more insulin sensitive. Importantly, we did not detect any significant differences in total body weight in Pdx1-Cre<sup>early</sup>;Smoflox/null mice when compared with controls, excluding the possibility that an decrease in body mass might lead to improved insulin sensitivity (supplemental Fig. 5A).

To determine whether insulin production is affected in Pdx1-Cre<sup>early</sup>;Smoflox/null pancreata, we analyzed Insulin expression levels. Our results showed that Insulin gene expression in islets was reduced by 40% (Fig. 6C), in accordance with Insulin protein levels from total pancreata that were diminished by the same amount (Fig. 6D). In contrast to previous studies demonstrating a positive role for Hh signaling on Insulin production through Pdx1
expression in INS1 cells, Pdx1 levels were not significantly altered in Pdx1-Creearly;Smoflox/null pancreata (Fig. 6C). While Insulin gene and protein levels were diminished, we did not detect any significant differences in fasting serum insulin levels (supplemental Fig. 5B). To assess insulin secretion defects, we treated isolated islets to conditions of low and high glucose and measured their corresponding insulin secretion output. Indeed, in isolated islets, we found impaired insulin secretion (Fig. 6E). However, when comparing the level of insulin secreted from low to high glucose levels in each group, control islets secreted two-and-a-half times the baseline level of insulin in high-
glucose conditions, while \textit{Pdx1-Cre\textsuperscript{early};Smoflox/null} islets secreted four times the baseline level of insulin. Thus, \textit{Pdx1-Cre\textsuperscript{early};Smoflox/null} islets secreted relatively more insulin than control islets, indicative of impaired insulin production rather than insulin secretion. To further rule out a secretion defect, we performed insulin secretion assays in the presence of potassium chloride (KCl), a known stimulator of insulin secretion. We found that in low glucose conditions with KCI, \textit{Pdx1-Cre\textsuperscript{early};Smoflox/null} islets secrete equivalent amounts of insulin as control islets (Fig. 6F). But when challenged in high-glucose conditions with KCl, \textit{Pdx1-Cre\textsuperscript{early};Smoflox/null} islets secrete comparatively less insulin than controls (Fig. 6F). Together, these results indicate that while mutant islets are capable of increasing secretion of insulin, they have deficiencies in meeting higher insulin demands due to diminished insulin production capacity. To further investigate the possible causes of the defects in insulin synthesis and secretion, we analyzed the expression of β-cell genes that are important for β-cell physiology. We found that gene expression for a number of β-cell genes, including \textit{Glut2}, \textit{Glucokinase}, and \textit{NeuroD1}, were normal (supplemental Fig. 4A). Gene expression analysis for secretory pathway components, including \textit{Kir6.2}, \textit{SUR1}, \textit{Calpain10}, and \textit{SNAP25}, were also normal (supplemental Fig. 4B).

Glucose intolerance caused by reduced insulin production may also be a result of reduced β-cell mass. To determine whether β-cell mass is altered in adult mice, we measured β-cell mass in \textit{Pdx1-Cre\textsuperscript{early};Smoflox/null} versus control mice. Surprisingly, we found that β-cell mass in \textit{Pdx1-Cre\textsuperscript{early};Smoflox/null} mice was actually increased nearly twofold (Fig. 6G). Collectively, these data indicate that \textit{Pdx1-Cre\textsuperscript{early};Smoflox/null} pancreata try to compensate for reduced insulin production levels by increasing β-cell mass. Considering these results, we conclude that adult \textit{Pdx1-Cre\textsuperscript{early};Smoflox/null} mice have normal pancreas morphology but impaired β-cell function due to reduced insulin production despite increased β-cell mass.

**DISCUSSION**

Understanding proper β-cell formation and function are imperative to finding treatments and cures for β-cell pathologies such as diabetes. Currently, it is accepted that Shh expression must be excluded from the budding mouse pancreatic epithelium. However, the subsequent expression of Hh signaling components later during pancreas formation suggest that there may be a functional requirement for Hh signaling during pancreas morphogenesis. In this study, we examined the requirement for epithelial Hh signaling in the developing pancreas. Apelqvist et al. (9) showed that \textit{Ptch1} is not expressed in e9.5 pancreatic epithelium. Through \textit{Ptch1-LacZ} staining, we show that \textit{Ptch1} expression is detectable by e10.5, indicating that Hh-active cells reside in the pancreatic epithelium soon after pancreas specification. While the number of \textit{Ptch1-LacZ\textsuperscript{+}} cells expands during development, the expression levels are low and positive cells are restricted to the ductal and endocrine compartments. In contrast, after birth, \textit{Ptch1-LacZ\textsuperscript{+}} cells stain robustly in the islets and ducts, suggesting that the level of Hh signaling after birth is higher than in utero.

During early stages, we also find some \textit{Ptch1-LacZ\textsuperscript{+}} cells in the neighboring pancreatic mesenchyme. Epithelial-mesenchymal crosstalk is important for proper organ formation, and Hh signaling may function differently in either compartments. To address the functional requirement of Hh signaling in the pancreas epithelium, we generated \textit{Pdx1-Cre\textsuperscript{early};Smoflox/null} mice, which sufficiently lose Smo expression and Hh signaling specifically in the epithelial compartment of the organ. The finding that Hh signaling is required for proper early epithelial expansion and branching in developing pancreata is contrary to previous studies (9–15) that suggested that Hh signaling inhibits mammalian pancreas growth. However, prior studies focused on ectopic activation of Hh ligand expression and the negative effects observed on pancreas development resulted from perturbing the epithelial-mesenchymal crosstalk required for proper organ formation. Moreover, previous studies emphasize that the inhibitory effects of Hh signaling is primarily responsible for establishing the pancreas organ boundaries in the foregut. Our results extend this model by demonstrating an additional role for low-level Hh signaling that promotes the early expansion of pancreatic epithelium by e12.5. Evidence for Hh signaling as mediator of cell proliferation has been broadly noted (4,5). Surprisingly, Hh activity appears to block proliferation of other pancreatic epithelial cells at midgestation, suggesting temporally distinct roles during pancreas formation. Thus, while our data point to Hh signaling as a mediator of cell proliferation during pancreas development, the exact mechanisms by which the pathway regulates epithelial proliferation in a transient and dynamic manner need to be explored further.

In addition to impaired pancreas morphogenesis, \textit{Pdx1-Cre\textsuperscript{early};Smoflox/null} mice have delayed β-cell development. This conclusion is based on the results that the insulin-positive β-cell area was reduced, but β-cell progenitor numbers were increased at e15.5, and the full complement of β-cells was established at the end of gestation. Moreover, albeit not conclusive, temporal analysis of \textit{Ngn3} expression indicated a trend whereby peak expression of \textit{Ngn3} was noted earlier in \textit{Ngn3\textsuperscript{–/–}} mice than in control mice. This suggests a temporary delay in general endocrine cell development in \textit{Pdx1-Cre\textsuperscript{early};Smoflox/null} mice that is overcome with time.

In support of this notion is the observation that the number of α-cells, which form earlier than β-cells during normal pancreas organogenesis, was unchanged at e15.5 compared with controls.

Our studies in mice complement Zebrafish work that has demonstrated the requirement for Hh signaling during gastrulation, a developmental stage that precedes the onset of organ formation. In Zebrafish, inhibition of Hh signaling at early gastrulation stages blocks the formation of pancreatic endocrine cells (37,38). Interestingly, this requirement for Smo function is non-cell autonomous (37). Later during gastrulation, inhibition of Hh signaling increases the formation of insulin-producing cells (39). While the relationship of Hh signaling during mouse gastrulation and subsequent pancreatic endocrine cell formation has not been elucidated, our present work and the studies in Zebrafish support the notion that the level and timing of Hh signaling need to be closely regulated to allow proper endocrine cell development.

Adult \textit{Pdx1-Cre\textsuperscript{early};Smoflox/null} mice possess normal pancreas morphology, while the β-cells are dysfunctional. Although these mice are sensitive to exogenous insulin and have an increased β-cell mass, their β-cells produce less insulin, resulting in reduced insulin secretion and a glucose intolerance phenotype. Importantly, our data indicate that the primary defect lies within the production of insulin whereas secretion appears intact. Thomas and
colleagues (16,17) showed that β-cell lines respond to Hh activity by increasing insulin production and secretion through regulation of Pdx1 expression. Although we did not detect significant changes in Pdx1 transcription by RT-PCR or in Pdx1 protein levels by qualitative staining in mutant mice, Insulin expression was reduced and isolated islets recapitulated decreased insulin secretion in culture. While further studies are needed, this suggests a mechanism independent of Pdx1 regulation on Insulin transcription for Hh-regulated insulin production. The differences observed between the previous cell culture experiments and our in vivo analysis maybe due to the inherent altered state of insulinoma cell lines versus the native β-cell.

Nevertheless, both studies emphasize the role of Hh signaling in maintaining proper insulin production. Further support comes from the finding that Ptc1-LacZ expression, indicative of Hh signaling activity, becomes dramatically stronger in postnatal islets at a time when the demand for β-cell functionality and insulin activity begins.

Interestingly, Pdx1-Creearly;Smoflox/null mice are more insulin sensitive. While we do not understand the mechanisms that result in this change, these data rule out impaired insulin sensitivity as a cause for glucose intolerance phenotypes in Pdx1-Creearly;Smoflox/null mice. Coupled with data demonstrating that insulin secretion is intact, these results emphasize that the primary defect in Pdx1-Creearly;Smoflox/null mice lies in insulin production in β-cells.

It should be noted that previous studies have linked Hh signaling to the formation and progression of pancreatic adenocarcinoma (40–47). During neoplastic transformation, excessive Hh ligands secreted from the tumorigenic epithelium (41,42) are likely to act predominately on the surrounding stroma in a paracrine manner (40,46,47). In the endocrine compartment, previous work (13,16) has shown expression of Hh ligands, Ihh and Dhh, in pancreatic islet cells. Unfortunately, due to the absence of agents suitable for cell type–specific expression analysis, the exact complement of those cells within the islets that produce and secrete ligands is currently missing. Thus, while our work and work from others indicate that epithelial-derived β-cells respond to Hh ligands in a juxtacrine or autocrine fashion, unequivocal resolution of this question awaits additional experiments with improved reagents.

As we struggle to find potential treatments and cures for diabetes, the need to generate or expand more functional β-cells remains unrequited. Understanding the mechanisms that will allow us to generate and sustain β-cell populations and function is imperative. Data presented here demonstrate that epithelial Hh signaling is important during pancreas development and in maintaining insulin production in the adult β-cell, thus adding another layer to the current perspective that views Hh inhibition as important for the generation of functional pancreas endocrine cells.

ACKNOWLEDGMENTS

Work in M.H.’s laboratory was supported by a grant from the National Institutes of Health (DK60533).

No potential conflicts of interest relevant to this article were reported.

Many thanks to Michael German and Didier Stainier, members of the Hebrok and German lab, for helpful criticisms and advice; Pao-Tien Chuang for reagents; Gregory Szot and the Islet Production Core Facility staff for their help with islet isolations; Fred Schaufele and the Diabetes Endocrinology Research Center Imaging facility for technical assistance and use of microscopes; the Cancer Center Genomic Facility for help with Fgf10 quantitative Taqman PCR; and the University of California San Francisco Biomedical Sciences Graduate Program.

Parts of this article were presented in poster form at the 68th Scientific Sessions of the American Diabetes Association, San Francisco, California, 6–10 June 2008.

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