Intra-Endodermal Interactions Are Required for Pancreatic β Cell Induction

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SUMMARY

The cellular origin of signals that regulate pancreatic β cell induction is not clearly defined. Here, we investigate the seeming paradox that Hedgehog/Smoothened signaling functions during gastrulation to promote pancreatic β cell development in zebrafish, yet has an inhibitory role during later stages of pancreas development in amniotes. Our cell transplantation experiments reveal that in zebrafish, Smoothened function is not required in β cell precursors. At early somitogenesis stages, when the zebrafish endoderm first forms a sheet, pancreatic β cell precursors lie closest to the midline; however, the requirement for Smoothened lies in their lateral neighbors, which ultimately give rise to the exocrine pancreas and intestine. Thus, pancreatic β cell induction requires Smoothened function cell-nonautonomously during gastrulation, to allow subsequent intra-endodermal interactions. These results clarify the function of Hedgehog signaling in pancreas development, identify an unexpected cellular source of factors that regulate β cell specification, and uncover complex patterning and signaling interactions within the endoderm.

INTRODUCTION

How pancreatic β cells arise from the endoderm is a question of great importance in our understanding and treatment of diabetes mellitus (DM). Recent efforts have been aimed at directing stem cells to differentiate in vitro into pancreatic β cells for use in transplantation therapy of Type 1 DM (D’Amour et al., 2006). Identifying the extrinsic cues responsible for inducing pancreatic β cells in vivo should facilitate these efforts (Murmaugh and Melton, 2003; Stainier, 2006).

During the past decade, interactions between the mesoderm and endoderm have been a major focus of studies of pancreas and pancreatic β cell induction. These studies have identified a role for the notochord (Hebrok et al., 1998) and vascular endothelium (Lammert et al., 2001) in inducing the pancreas from the endoderm. Also, signals from the lateral plate mesoderm in chick (Kumar et al., 2003) and presomitic mesoderm in zebrafish (Staf ford et al., 2006) have been implicated in patterning the endoderm in the anterior-posterior (A-P) axis, thereby determining the A-P location of the pancreas. After the pancreas is initially induced from the endoderm, the adjacent, or pancreatic, mesenchyme regulates subsequent steps of pancreatic β cell formation by inducing the proliferation of pancreatic progenitors (Attali et al., 2007) and repressing the premature differentiation of pancreatic β cells (Miralles et al., 1998). However, besides Notch signaling, which has been suggested to act within the pancreatic epithelium (Apelqvist et al., 1999; Jensen et al., 2000; Jensen, 2004; Zecchin et al., 2007), the potential role of intra-endodermal interactions in pancreatic β cell induction has not been adequately investigated.

As in amniotes, the zebrafish pancreas is formed from multiple buds (Field et al., 2003). However, unlike what is observed in amniotes, the dorsal pancreatic bud in zebrafish is thought to give rise exclusively to endocrine cells (Field et al., 2003), thereby facilitating the study of endocrine differentiation. Expression of the homeodomain transcription factor gene, pdx1, the earliest pancreatic marker, is first detected by in situ hybridization at the 10-somite stage (14 hr post fertilization [hpf]) in broad bilateral populations of anterior endodermal cells (Biernar et al., 2001). At the 12-somite stage (15 hpf), insulin expression appears in a medial subset of the pdx1-positive cells (Biernar et al., 2001). As these dorsal pancreatic β cells mature, they aggregate to form a single islet cluster by 24 hpf.

In zebrafish, specification of the dorsal pancreatic endocrine cells requires Hedgehog (Hh) signaling (Roy et al., 2001; dilorio et al., 2002). Zebrafish embryos homozygous for loss-of-function mutations in sonic hedgehog a (shha) or smoothed (smo) display a nearly complete absence of pancreatic endocrine expression of pdx1, islet1, neurod, nkd2.2, and insulin (Roy et al., 2001; dilorio et al., 2002). These data have generated confusion about the role of Hh signaling in pancreas development since it has been shown in amniotes that repression of Hh ligand expression in the endoderm is necessary for pancreas induction (Hebrok et al., 1998; Hebrok, 2003). In mouse, misexpressing Shh in the embryonic pancreatic region, by using the Pdx1 promoter, perturbs pancreas development (Apelqvist et al., 1997), while Shh+/−/Ihh+/− mice show increased pancreatic mass and endocrine differentiation (Hebrok et al., 2000). Also, treating chick embryonic endodermal explants with cyclopamine to block Smo function led to ectopic pancreatic bud and endocrine formation (Kim and Melton, 1998). This apparent difference in the requirement of Hh signaling during pancreas development in zebrafish and amniotes may be due to differences between organisms (Roy et al., 2001; dilorio et al., 2002). However, since Smo mutant mice die before the pancreas develops (Zhang et al., 2001) and most of the ex vivo analyses in amniotes have been done at post-gastrulation stages (Hebrok et al., 1998; Kim and Melton, 1998),...
a direct comparison of Smo function in zebrafish and mouse pancreatic development has not yet been possible, leaving this seeming paradox open to alternative explanations (d'Ilorio et al., 2002).

Here, we find that Smo function is not required cell-autonomously for pancreatic β cell induction in zebrafish, a result which may help resolve this paradox. However, we show that Smo function is required during gastrulation in precursors of laterally located endodermal cells for the induction of medially located cells into pancreatic β cells. We further show by lineage tracing that the medially located endodermal cells between somites 1 and 4 give rise exclusively to pancreatic endocrine cells, while the lateral cells immediately adjacent to these medial cells give rise mainly to the exocrine pancreas and intestine. Taken together, these data provide evidence that intra-endodermal interactions are critical for the induction of pancreatic β cells.

RESULTS

Initial Appearance of Pancreatic β Cells in the Context of Adjacent Tissues

To identify tissue interactions potentially responsible for inducing pancreatic β cells, we examined transverse sections of Tg(ins:GFP) embryos (Huang et al., 2001). Tg(ins:GFP) expression is first detected in the endoderm at the 12-somite stage (15 hpf) (Figure 1A), lateral to the notochord and medial to the somites (Figures 1B and 1C). At this stage, the endoderm consists of a monolayer of cells directly apposed to various mesodermal derivatives such as the notochord and somites (Figure 1C). However, the lateral plate mesoderm (LPM) has not yet migrated to the midline and remains lateral to the somites until the 18-somite stage (18 hpf) (Figure 1C and data not shown). At the 15-somite stage (16.5 hpf), while Tg(ins:GFP)-expressing cells remain lateral to the somites, gaps (asterisks) can be observed in the midline (Figure 1E and data not shown). At the 18-somite stage, the bilateral Tg(ins:GFP)-expressing cells have started to migrate medially along with other endodermal cells, and are aggregating to form a single cluster (Figures 1G and 1H). To better visualize pancreatic β cells in the context of the endodermal sheet, we examined Tg(ins:GFP);Tg(sox17:DsRed)embryos at the 12-somite stage, the endodermal sheet has not yet completely fused to the midline (Figure 1F), and Tg(ins:GFP)-expressing cells are located at the medial edges of the bilateral populations (Figure 1F). At the 15-somite stage, there are still gaps in the anterior endodermal sheet, and most Tg(ins:GFP)-expressing cells remain lateral to the notochord (Figure 1).

Hedgehog Signaling Is Required for the Induction of Dorsal Pancreatic β Cells in Zebrafish

Induction of dorsal pancreatic bud-derived β cells is severely impaired in zebrafish embryos lacking Hh signaling (Roy et al., 2001; d’Ilorio et al., 2002). Since Smo, a seven-pass transmembrane protein, is essential for transducing the Hh signal...
cell-autonomously (Ingham and McMahon, 2001), we used a null allele of smo (Chen et al., 2001) for our analyses.

At 11 hpf, the Tg(sox17:DsRed)s903-expressing endodermal cells show a similar arrangement and distribution around the midline (asterisks) in wild-type (A) and smo mutants (B). Cells in the anterior neural tube (arrowhead) also express the ins:GFP transgene. (E) Tg(ins:GFP)-expressing cells form a cluster in 24 hpf wild-type embryos (arrow). (F) Tg(ins:GFP)-expressing cells are completely missing in most smo mutants, though in approximately 5% of the mutants (n > 100 mutants examined), one or two Tg(ins:GFP)-expressing cells can be observed (F, arrow).

(G–J) Confocal projections of wild-type (G and I) and smo mutant (H and J) endoderm at 34 (G and H) and 96 (I and J) hpf. (G and H) Tg(gutGFP)s854 embryos were stained for Islet1 (red) and Insulin (blue). (G) The endodermal cells (green) form a solid multicellular rod, and pancreatic endocrine cells (arrow and inset) aggregate into one cluster in wild-type embryos. (H) In smo mutants, the endodermal cells fail to condense into a midline rod, and pancreatic endocrine cells are completely absent. (I and J) Tg(ins:GFP) embryos were stained for pan-Cadherin (red) and 2F11 (blue) to mark the endoderm and ductal structures, respectively. (I) Tg(ins:GFP)-expressing β cells (arrow) form a cluster inside the wild-type pancreas. (J) Although the morphology of the pancreas and liver is disrupted, Tg(ins:GFP)-expressing β cells (arrows) appear in smo mutants in ventral pancreatic bud derived tissues. L, liver; P, pancreas; IB, intestinal bulb.

To determine the specificity of the absence of dorsal pancreatic β cells in smo mutants, we examined pdx1 expression as well as the formation of ventral pancreatic bud derived β cells in smo mutants. Consistent with previous studies (Roy et al., 2001; diIorio et al., 2002), we found that, in smo mutants, insulin expression at 18 and 24 hpf (Figures S1A–S1D; see the Supplemental Data available with this article online) as well as the high-level expression of pdx1 at 18 hpf (Figures S1E and S1I) are completely absent, while the low-level expression of pdx1 is still present at 18 hpf (Figure S1I) in a restricted domain of the developing gut (Figures S1F–S1H and S1J–S1L). Furthermore, although dorsal pancreatic β cells are completely absent in smo mutants at 34 hpf (Figure 2H) as compared to wild-type (Figure 2G), ventral pancreatic bud derived β cells appear in smo mutants at later stages of development (Figures 2I and 2J). These data, together with previous studies showing relatively unaffected exocrine pancreas formation in Hh mutants (diIorio et al., 2002; Zecchin et al., 2004), indicate that Smo function is required specifically for the formation of dorsal pancreatic β cells.
Shh Compensates for the Loss of Axial Mesoderm in the Formation of Pancreatic Endocrine Cells

From early gastrulation stages in zebrafish, Hh ligands are expressed in the dorsal gastrula organizer, also known as the shield (Ertzer et al., 2007). During gastrulation, cells of the dorsal organizer extend in the A-P orientation and form the axial mesoderm, including the notochord. Until 24 hpf, when Hh ligand gene expression starts to be detected in the endoderm (Roy et al., 2001), the axial mesoderm acts as the major source of Hh ligands. Since Tg(ins:GFP)-expressing cells appear in the endodermal cells that are closest to the axial mesoderm (Figure 1C), this tissue could play important roles in inducing pancreatic endocrine cells. Indeed, it has been shown that floating head (flh) mutants, in which the axial mesoderm is missing (Talbot et al., 1995), exhibit a severe defect in pancreatic endocrine cell induction (Biemar et al., 2001). Therefore, to test the hypothesis that the axial mesoderm induces pancreatic endocrine cells via Hh production, we analyzed whether Hh overexpression in flh mutants was sufficient to replace the function of the axial mesoderm in pancreatic β cell induction. At 18 hpf, wild-type embryos show clusters of Tg(ins:GFP)-expressing cells near the midline (Figure 3A). However, Tg(ins:GFP) expression is completely absent in flh mutants (Figure 3B), although they show wild-type-like endodermal sheet formation. Interestingly, we found that when shh mRNA was injected at the one-cell stage, Tg(ins:GFP) expression was restored in shh-overexpressing flh mutants and scattered within the endodermal sheet (red). Most endodermal cells failed to converge following shh overexpression, leading to the presence of gaps (asterisks) within the endodermal sheet. Shh was shown to be required during gastrulation for the differentiation of pancreatic endocrine cells (Figure 1C).

Hedgehog Signaling Is Required during Gastrulation for the Differentiation of Pancreatic Endocrine Cells

Next, we asked when Hh signaling from the axial mesoderm was required for inducing pancreatic β cells by activating Hh
Developmental Cell

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Figure 4. Cell-Nonautonomous Requirement of Smoothened Function in the Induction of Dorsal Pancreatic Endocrine Cells

(A) Schematic diagram of the cell transplantation protocol. cas-overexpressing wild-type or smo mutant Tg(ins:GFP) donor cells were transplanted into wild-type hosts.

(B–G) Wild-type hosts with wild-type (B, D, and F) and smo mutant (C, E, and G) Tg(ins:GFP) donor cells at 30 hpf: dorsal views (B and C) and confocal projections (D–G). All donor cells are labeled with rhodamine dextran (red). Note that only donor cells can express the ins:GFP transgene. (B) Wild-type donor cells contribute to the endoderm and express the ins:GFP transgene in wild-type hosts (arrow; 52%, n = 77/147). (C) smo mutant donor cells contribute to the endoderm and express the ins:GFP transgene (arrow) in wild-type hosts as frequently as wild-type cells do (51%, n = 25/49). (D–G) Hosts were stained for Islet1, which marks all pancreatic endocrine cells (blue) and motoneurons (blue, asterisk). (D and F) Wild-type donor cells differentiate into Islet1-positive endocrine cells (red cytoplasm, blue nucleus; arrowhead), and many of the donor cells coexpress the ins:GFP transgene (triple-positive cells appear white; arrows). (E and G) smo mutant donor cells also differentiate into Islet1-positive signaling in flh mutants. To activate Hh signaling at specific time points, we used the Smo agonist purmorphamine (Sinha and Chen, 2006). Purmorphamine, a purine derivative, activates Hh signaling by directly binding Smo (Sinha and Chen, 2006). When purmorphamine was added to flh mutants at the onset of gastrulation (6 hpf), Tg(ins:GFP)-expressing cells were rescued (Figures S2C and S2H) to a level comparable to wild-type (Figures S2A and S2F). However, the number of rescued Tg(ins:GFP)-expressing cells in flh mutants decreased when purmorphamine was added at 8 (Figures S2D and S2I) and 10 (Figures S2E and S2J) hpf. When purmorphamine was added after the end of gastrulation (11 hpf), Tg(ins:GFP) expression was not rescued (data not shown) and appeared similar to the untreated flh mutants (Figures S2B and S2G). Together with previous data obtained from treating wild-type embryos with the Smo antagonist cyclopamine (diIorio et al., 2002), these data indicate that Hh signaling is required during gastrulation for inducing dorsal pancreatic endocrine cells.

Smoothened Function Is Not Required Cell-Autonomously for the Induction of Dorsal Pancreatic Bud Derived Endocrine Cells

Since prior studies in amniotes indicate that Hh signaling is not necessary and in fact needs to be blocked at the time of pancreas induction (Kim and Melton, 1998; Hberek et al., 2000; Hebrok, 2003), we hypothesized that Smo function was not required cell-autonomously for pancreatic β cell induction in zebrafish. To test this hypothesis, we analyzed the cell autonomy of the dorsal pancreatic β cell defect in smo mutants by cell transplantation. To target donor cells to the endoderm, we co-jected cas mRNA with the lineage tracer rhodamine dextran into Tg(ins:GFP)/smo−/− donors (Figure 4A). Overexpression of cas can induce sox17 expression in nonendodermal cells (Kikuchi et al., 2001; Sakaguchi et al., 2001) and direct cells not fated to become endoderm into the endodermal lineage (Dickmeis et al., 2001; Kikuchi et al., 2001; Sakaguchi et al., 2001; Stafford et al., 2006). After transplanting smo mutant cells into wild-type embryos, we analyzed the hosts at 30 hpf (Table S1A). We found that smo mutant donor cells gave rise to Tg(ins:GFP)-expressing cells (Figure 4C; 51%, n = 25/49) as frequently as wild-type cells did (Figure 4B; 52%, n = 77/147). Notably, smo mutant cells differentiated into Islet1-positive endocrine cells and expressed Tg(ins:GFP) (Figures 4E and 4G and Figures S3K–S3T) to a similar extent as wild-type cells did (Figures 4D and 4F and Figures S3A–S3J). These data indicate that Smo function is not required cell-autonomously for the induction of dorsal pancreatic endocrine cells in zebrafish.

Endodermal Requirement of Smoothened Function for Dorsal Pancreatic β Cell Induction

These findings imply that during β cell induction in the dorsal pancreatic bud, Smo function is required cell-nonautonomously, and they led us to investigate the identity of the tissues requiring Smo function for this process. Initially, we hypothesized that the nonendodermal defects in smo mutants were responsible for the endocrine cells (red cytoplasm, blue nucleus; arrowhead), and many of the donor cells coexpress the ins:GFP transgene (triple-positive cells appear white; arrows). Single- and double-channel images are shown in Figure S3.
Intra-Endodermal Interactions in β Cell Induction

To investigate the identity of the Smo-requiring endodermal cells in pancreatic β cell induction, we transplanted cas-overexpressing wild-type Tg(ins:GFP); smo−/− embryos into Tg(ins:GFP); smo−/− embryos (Figure 6A and Table S1D). When wild-type cells gave rise to the endoderm, Tg(ins:GFP) expression was fully restored in smo mutant hosts (Figure 6C; 44.5%, n = 48/108). Most of the Tg(ins:GFP) expression in smo mutant hosts occurred in wild-type donor cells (Figure 6D). However and importantly, when we transplanted a small number of donor cells, we found several experimental embryos (n = 6/48) where wild-type lateral endoderm correlated with Tg(ins:GFP) expression in medially located smo mutant host endoderm (Figure 6E). These data show that Smo function is required in laterally located endodermal cells for medially located cells to differentiate into pancreatic β cells. In addition, in five of those six embryos, wild-type endodermal cells were in direct contact with the Tg(ins:GFP)-expressing smo mutant cell(s), suggesting that contact or proximity between endodermal cells might be required for pancreatic β cell induction.

To further investigate whether direct cell-cell contacts are required for pancreatic β cell induction, we injected lower concentrations of cas MO (−0.1–0.4 ng) into Tg(ins:GFP); Tg(sox17; DsRed)−/− embryos to lower the number of endodermal cells and thus reduce the frequency of endodermal cell-cell contacts. (During gastrulation, endodermal cells are distributed in a salt-and-pepper fashion; Coutelle et al., 2001), and the lateral floor plate (LFP) cells (Odenthal et al., 2000) fail to differentiate due to their cell-autonomous requirement of Smo function (Hirsinger et al., 2004; Schafer et al., 2005). Since these two tissues are located adjacent to the Tg(ins:GFP)-expressing cells when these cells first appear (Figure 1C), they might be responsible for their induction. To test this hypothesis, we generated genetically mosaic embryos where wild-type cells were transplanted into Tg(ins:GFP); smo−/− embryos (Figure S4A and Table S1B). After transplantation, we analyzed the hosts at 18 hpf, a stage when the relative position of the endoderm and surrounding tissues is comparable to when Tg(ins:GFP) expression first appears. When wild-type donor cells differentiated into adaxial (Figures S4C and S4F, arrowhead) or LFP (Figures S4D and S4G, arrowhead) cells, Tg(ins:GFP) expression was in fact not rescued in smo mutant hosts. These data suggest that the adaxial and LFP cells are not the primary source of the Smo-dependent signal required for pancreatic β cell induction, and that another cell type requires Smo function for this process.

Surprisingly, we found in these transplantation experiments one embryo in which Tg(ins:GFP) expression in smo mutant endoderm was rescued when wild-type donor cells were incorporated into the endoderm directly adjacent to the rescued Tg(ins:GFP)-expressing cell (Figure S5). This finding suggested that it might be the endodermal cells adjacent to β cell precursors that require Smo function for pancreatic β cell induction. To test this hypothesis, we first asked whether the defects that lead to the absence of dorsal pancreatic β cells in smo mutants lie in endodermal or nonendodermal cells. To answer this question, we transplanted cas-overexpressing Tg(ins:GFP); smo−/− cells into cas MO-injected wild-type embryos (Figure 5A and Table S1C). cas MO-injected embryos completely lack all endoderm (Kikuchi et al., 2001; Sakaguchi et al., 2001), thus allowing the complete replacement of the host endoderm by donor-derived cells (Stafford et al., 2006). When the endoderm in wild-type hosts was derived from wild-type donor cells, the hosts showed wild-type-like Tg(ins:GFP) expression (Figures 5B and 5D; n = 74). However, when the endoderm in wild-type hosts was derived from smo mutant donor cells, Tg(ins:GFP) expression was completely absent in most cases (Figure 5C; 92%, n = 23/25), or was present in one or two cells (Figure 5E; 8%, n = 2/25). The absence of Tg(ins:GFP) expression in wild-type hosts with smo mutant endoderm together with our previous data (Figure 4) indicate that while pancreatic β cell precursors do not require Smo function cell-autonomously, Smo function in some other endodermal cells is required for pancreatic β cell induction.

Figure 5. Smoothened Function Is Required in the Endoderm for the Induction of Dorsal Pancreatic β Cells
(A) Schematic diagram of the cell transplantation protocol. cas-overexpressing wild-type or smo mutant Tg(ins:GFP) donor cells were transplanted into cas MO-injected wild-type hosts.
(B–E) Dorsal views of the cas MO-injected hosts with wild-type (B and D) and smo mutant (C and E) donor cells at 24 hpf. Donor cells are labeled with rhodamine dextran (red). All the endodermal cells of the hosts are replaced by donor cells. (B and D) Wild-type-like Tg(ins:GFP) expression in the endoderm derived from wild-type donor cells (95%, n = 74/78). (C and E) Tg(ins:GFP)-expressing cells are completely absent (IC, 92%, n = 23/25) or amount to one or two cells (IE, arrow: 8%, n = 2/25) when smo mutant donor cells form the endoderm in cas MO-injected wild-type hosts.

absence of the dorsal pancreatic endocrine cells. In smo mutants, the adaxial cells, which originate in the medial position of the somites (Coutelle et al., 2001), and the lateral floor plate (LFP) cells (Odenthal et al., 2000) fail to differentiate due to their cell-autonomous requirement of Smo function (Hirsinger et al., 2004; Schafer et al., 2005). Since these two tissues are located adjacent to the Tg(ins:GFP)-expressing cells when these cells first appear (Figure 1C), they might be responsible for their induction. To test this hypothesis, we generated genetically mosaic embryos where wild-type cells were transplanted into Tg(ins:GFP); smo−/− embryos (Figure S4A and Table S1B). After transplantation, we analyzed the hosts at 18 hpf, a stage when the relative position of the endoderm and surrounding tissues is comparable to when Tg(ins:GFP) expression first appears. When wild-type donor cells differentiated into adaxial (Figures S4C and S4F, arrowhead) or LFP (Figures S4D and S4G, arrowhead) cells, Tg(ins:GFP) expression was in fact not rescued in smo mutant hosts. These data suggest that the adaxial and LFP cells are not the primary source of the Smo-dependent signal required for pancreatic β cell induction, and that another cell type requires Smo function for this process.
and pepper pattern [Alexander and Stainier, 1999].) In 18 hpf cas MO-injected embryos, the continuous endodermal layer observed in wild-type (Figure S6A) fails to form (Figure S6B). In a majority (n = 53/67; Table S4) of cas MO-injected embryos, the endodermal cells were located laterally and Tg(ins:GFP) expression was absent (Figure S6B). However, in the other 14 embryos, a few endodermal cells had migrated close to the midline, and some of them were expressing the ins:GFP transgene (Figures S6C and S6D). Interestingly, in 4 of these 14 embryos, a single Tg(ins:GFP)-expressing cell was located close to, but not in direct contact with, other endodermal cells (Figure S6D). These data indicate that continuously direct endodermal cell-cell contacts are not essential to induce Tg(ins:GFP) expression.

Next, in order to examine how many Tg(ins:GFP)-expressing smo mutant cells can be rescued by wild-type endodermal cells, we performed the same transplantation experiment as shown in Figure 6A (wild-type endodermal cells into smo mutant hosts), but this time, we used wild-type embryos that do not contain the ins:GFP transgene as donors (Figure 6F and Table S2). In this way, the Tg(ins:GFP)-expressing smo mutant cells could be easily identified, since only the hosts contain the ins:GFP transgene. When we analyzed smo mutant hosts at 30 hpf, we found several experimental embryos (n = 7/26) that showed rescued Tg(ins:GFP) expression in proximity to wild-type endodermal cells (Figure 6H). When wild-type donor cells were incorporated into nonendodermal regions or the posterior part of the gut, Tg(ins:GFP) expression was not rescued (Figure 6G). Notably, the number of rescued Tg(ins:GFP)-expressing cells was more than four (four to seven) in all seven examples. In contrast, in smo mutants without endodermal incorporation of wild-type cells, Tg(ins:GFP) expression was completely absent in most of the cases or present at most in two cells (5%, n = 2/44), similar to what was observed in other experiments (Figures 2F and 5E). Altogether, these data show that intra-endodermal interactions are required for pancreatic β cell induction.

**Lineage Tracing Reveals Distinct Fates between the Medial and Lateral Endodermal Cells**

Next, we investigated the fate of the lateral endodermal cells, those that require Smo function for the differentiation of their medial neighbors into pancreatic β cells (Table S3). To mark endodermal cells with single-cell resolution, we used a transgene. When we analyzed的文章内容。
photoactivatable DMNB-caged fluorescein dextran conjugate as a lineage tracer (Keegan et al., 2005; Vogeli et al., 2006). In addition, we used the Tg(sox17:GFP)s870 line to visualize the endodermal sheet in live embryos at the 6- to 8-somite stage (Figure 7B) and to target specific endodermal cells with a 405 nm laser. Since Tg(ins:GFP)-expressing cells appear in the region between somites 1 and 4, we marked endodermal cells within somites 2 and 3 (Figures 7A and 7B). In each experimental embryo, we labeled two endodermal cells at each of three specific positions in the medio-lateral axis: the most medial cells (medial, dark green), cells immediately adjacent to the medial cells (lateral 1, green), and cells one cell apart from the medial cells (lateral 2, light green). Brackets indicate the area where uncaging was performed. (D–F) Ventral confocal images of control embryos at the 10-somite stage, confirming the accuracy of endodermal targeting by the laser. Tg(sox17:GFP)s870 embryos were stained for GFP (gray) and uncaged fluorescein (red). Two endodermal cells in the medial (D), lateral 1 (E), and lateral 2 (F) positions were labeled in the region between somites 2 and 3. (G–L) Confocal projections of Tg(sox17:GFP)s870 embryos at 50 hpf showing the progeny of the medial (G and J), lateral 1 (H and K), and lateral 2 (I and L) cells. Tg(sox17:GFP)s870 embryos were stained for GFP (green), Insulin (blue), and uncaged fluorescein (red). (G–J), (G and J), (H and L), and (I and K) are the same embryos. (G and J) Medial cells gave rise exclusively to pancreatic endocrine cells (arrows). (H and K) Lateral 1 cells gave rise to exocrine cells (double arrows), a small number of endocrine cells (arrows), as well as intestinal tissue (arrowhead) adjacent to the pancreas. (I and L) Lateral 2 cells gave rise to liver cells (asterisks), intestine (arrowhead), and exocrine cells (double arrow). Lateral 2 cells showed very little, if any, contribution to pancreatic endocrine cells (arrow).

**DISCUSSION**

Altogether, our data indicate that Smo function is required in precursors of laterally located endodermal cells for pancreatic β cell...
induction in precursors of their medial neighbors. Data from the purmorphamine treatment and previous studies with cyclopamine (diIorio et al., 2002) show that Hh signaling is required during early gastrulation for pancreatic β cell induction in zebrafish. Using cyclopamine to block Smo function, diIorio et al. (2002) found that treating embryos at the onset of gastrulation ablates dorsal pancreatic endocrine cells, while treatment after the end of gastrulation results in a wild-type sized but anteriorly shifted islet. At the early gastrulation stage, the only documented source of Hh ligands is the dorsal organizer (diIorio et al., 2002), which gives rise to the axial mesoderm. Also, it should be noted that in zebrafish, hh expression in the endoderm is first evident at 24 hpf (Roy et al., 2001), long after dorsal pancreatic endocrine cells are induced. Therefore, we propose a model whereby during gastrulation, Hh ligands secreted from the dorsal organizer and its derivatives are received directly by endodermal cells, some of which require Smo function to induce, or allow the differentiation of, neighboring endodermal cells into pancreatic β cells.

The Axial Mesoderm Induces Pancreatic Endocrine Cells via Hh Production

The absence of Tg(ins:GFP) expression in flh mutants, which lack axial mesoderm from early gastrulation stages, confirms the important role of this tissue in inducing pancreatic endocrine cells, as previously suggested (Bieler et al., 2001). Since the axial mesoderm produces a variety of signaling molecules in addition of Hh (Hebrok et al., 1998; Hebrok, 2003), its exact role in pancreatic endocrine induction has been unclear. Here, we show that the absence of pancreatic endocrine cells in flh mutants can be rescued by overexpressing Shh or activating Smo with purmorphamine, indicating that the axial mesoderm functions in pancreatic endocrine cell induction via its secretion of Hh ligands.

Dual Role of Hh Signaling in Zebrafish Pancreas Development

Previous studies in amniotes have shown that after gastrulation has occurred and organ boundaries have formed, Hh ligand expression in the prepancreatic epithelial cells needs to be suppressed for pancreas development to proceed (Apelqvist et al., 1997; Kim and Melton, 1998; Hebrok et al., 2000; Hebrok, 2003). However, due to the early lethality of mouse mutants lacking all Hh signaling, the requirement of this pathway for pancreas development has not been determined. Using genetic mosaic analyses in zebrafish, we have shown that smo mutant donor cells are able to differentiate into dorsal pancreatic endocrine cells in wild-type hosts. Also, our data showing that ventral pancreatic bud derived β cells appear in smo mutants at later stages of development confirm that cells lacking Smo function can differentiate into pancreatic β cells. These findings provide conclusive genetic evidence that endodermal cells do not require Hh signaling cell-autonomously during their differentiation into pancreatic endocrine cells. However, the early cell-nonautonomous requirement of Hh signaling in inducing dorsal pancreatic endocrine cells in zebrafish indicates that Hh signaling acts in at least two different ways during pancreas development. First, and as mentioned earlier, Hh from the dorsal organizer is required by gastrulating endodermal cells for pancreatic endocrine cell induction. Second, Hh ligand expression in the endoderm starting at 24 hpf may help to set up organ boundaries. For example, in zebrafish tcf2 mutants where shh expression is expanded into the pancreatic endodermal region, pdx1 expression is severely reduced (Sun and Hopkins, 2001). Also, in zebrafish embryos with lower Meis3 or Pbx4 function, shh expression is reduced in the anterior endoderm, and pancreatic endocrine cells are shifted to this anterior endoderm (diIorio et al., 2007). Thus, it appears that at late stages, Hh signaling has inhibitory effects on pancreas development in both amniotes and zebrafish. It will be interesting to determine whether the early cell-nonautonomous requirement for Hh signaling observed during β cell induction in zebrafish is also present in amniotes.

Intra-Endodermal Interactions for Inducing Dorsal Pancreatic β Cells

In an attempt to identify the cells responsible for mediating the cell-nonautonomous requirement of Hh signaling in pancreatic endocrine cell induction, we observed that cell-cell interactions between endodermal cells appear to be essential for this process. This result was unexpected because mesodermal cells have been assumed to be largely responsible for inducing the differentiation of pancreatic β cells (Hebrok et al., 1998; Lammert et al., 2001; Kumar et al., 2003; Stafford et al., 2006). Of course, it remains possible that the intra-endodermal interactions might be mediated indirectly through neighboring mesodermal cells, although the observation that, in a majority of cases, wild-type endodermal cells were in close contact with the rescued smo mutant cells makes this possibility unlikely. When wild-type cells were more than two cells apart from medial cells, Tg(ins:GFP) expression in smo mutant cells was not rescued (data not shown), suggesting that the intra-endodermal interactions require direct cell-cell contact or act at a short range. To further explore this hypothesis, we examined embryos with a lower number of endodermal cells. The presence of isolated pancreatic β cells in these embryos indicates that continuously direct endodermal cell-cell contacts are not required for pancreatic β cell induction. However, new tools such as the generation of a membrane-bound fluorescent transgene to visualize endodermal cells will be required to analyze the extent of transient cell-cell contacts during gastrulation and early segmentation stages. Notch signaling acts within the pancreatic endoderm (Apelqvist et al., 1999; Jensen et al., 2000; Zecchin et al., 2007) and needs direct cell-cell contact (Beatus and Lendahl, 1998), and thus may be part of the interactions reported here. However, this hypothesis is unlikely because inhibiting Notch signaling in zebrafish results in excessive differentiation of pancreatic β cells from pdx1-positive progenitors (Zecchin et al., 2007). And when Notch signaling is activated, pdx1 expression is maintained while further differentiation into pancreatic endocrine cells is blocked (Zecchin et al., 2007). Therefore, inhibition or activation of Notch signaling does not resemble the pancreas phenotype of smo mutants, i.e., absence of insulin expression and absence of the high-level pdx1 expression, indicating that Notch signaling by itself does not account for the intra-endodermal interactions uncovered in these studies. Moreover, when we treated smo mutants at different developmental stages with DAPT, a gamma-secretase inhibitor that blocks the Notch signaling pathway (Geling et al., 2002), Tg(ins:GFP) expression was not rescued (data not shown).
Intra-Endodermal Interactions in β Cell Induction

Medio-Lateral Patterning of the Endodermal Sheet in Zebrafish

To better understand the role of the lateral endodermal cells in pancreatic β cell induction, we investigated their fate by labeling them at a stage just prior to the onset of pdx1 expression. Previous fate maps of the zebrafish endoderm were generated by labeling cells at pregastrulation stages (Warga and Nüsslein-Volhard, 1999; Ward et al., 2007), and these studies revealed that endocrine progenitors are located more dorsally than exocrine progenitors in early gastrula-stage embryos (Ward et al., 2007). We found that endodermal cells in the same A-P level give rise to different tissues based on their position in the medio-lateral axis. Medial cells gave rise exclusively to pancreatic endocrine cells; lateral 1 cells gave rise to pancreatic exocrine cells and a small number of endocrine cells, as well as to intestinal tissue adjacent to the pancreas; lateral 2 cells gave rise to the liver as well as the exocrine pancreas and adjacent intestine. It is interesting to note that pdx1 expression at mid-somitogenesis stages appears to cover at least the medial and lateral 1 cells (Biemar et al., 2001). After the endodermal cells converge to the midline and the organs, such as the liver and ventral pancreas, bud out, pdx1 expression can be detected in the dorsal and ventral pancreas as well as the intestinal bulb (Field et al., 2003), tissues comprising the progeny of lateral 1 cells. Since our transplantation data indicate that the intra-endodermal interactions between the medial and lateral cells require close contacts, we propose that pdx1-expressing lateral 1 cells, or their precursors, induce their neighboring pdx1-expressing medial cells, or their precursors, to differentiate into pancreatic endocrine cells.

Possible Modes of Intra-Endodermal Interactions

It will be interesting to determine the molecular mechanisms for the function of the lateral endoderm in pancreatic β cell induction, and the following considerations may help unravel these mechanisms. First, the lateral endoderm, which has presumably been exposed to medium-to-low levels of Hh ligands from the axial mesoderm during gastrulation, induces neighboring endodermal cells into pancreatic β cells. Previous fate map data (Warga and Nüsslein-Volhard, 1999; Ward et al., 2007) suggest that at early gastrulation stages, the progenitors of pancreatic endocrine cells are located closer to the source of Hh ligands than the progenitors of the exocrine pancreas and intestine. Therefore, in this model, medium-to-low levels of Hh ligands turn on secondary signals in certain endodermal cells, which subsequently induce the differentiation of neighboring cells into pancreatic β cells. It is of course possible that these intra-endodermal interactions can be mediated by signals that can act in an autocrine as well as paracrine fashion. In fact, the presence of isolated, single Tg(ins:GFP)-expressing cells in a few cas MO-injected embryos (Figure S6D) suggests the possibility of autocrine signaling. By 16 hpf, Wnt (Ober et al., 2006) and Bmp (Shin et al., 2007; C. Shin and D.Y.R.S., unpublished data) signals necessary for liver induction are expressed by the lateral plate mesoderm, which is located close to the lateral 2 cells. Mechanisms required for pancreatic β cell induction may include blocking these Wnt and Bmp signals. In addition, while our studies reveal the importance of intra-endodermal interactions in pancreatic β cell induction, they do not exclude a role for mesodermal signals.

Delineating the precise cell-cell interaction requirements during pancreatic β cell induction is an initial step toward a complete mechanistic understanding of this process. Identification of intra-endodermal interactions critical for pancreatic β cell induction within the pdx1-positive domain should facilitate the isolation of the signals themselves and the in vitro production of pancreatic β cells from stem cells.

EXPERIMENTAL PROCEDURES

Zebrafish Strains

Embryos and adult fish were raised and maintained under standard laboratory conditions. We used the following mutant and transgenic lines: smo<sup>1640</sup> (Chen et al., 2001), fh<sup>11</sup> (Talbot et al., 1995), Tg(ins:GFP) (Huang et al., 2001), Tg(sox17:GFP<sup>AV</sup>)<sup>AV</sup>70, Tg(sox17:DsRed<sup>AV</sup>)<sup>AV</sup>03, and Tg(gutGFP<sup>AV</sup>)<sup>AV</sup>854 (Field et al., 2003).

Details about the Tg(sox17:GFP<sup>AV</sup>)<sup>AV</sup>70 and Tg(sox17:DsRed<sup>AV</sup>)<sup>AV</sup>03 lines and genotyping of smo<sup>AV</sup>1640 fish can be found in the Supplemental Data.

Embryo Microinjection and Transplantation

Sense-strand-capped cas and shh mRNA were synthesized with mMESSAGE mMACHINE (Ambion). For shh overexpression, embryos were injected with 200 pg of shh mRNA. For transplantation, donor embryos were injected with 2.5% rhodamine dextran (tetramethylrhodamine dextran, 70,000 MW, lysine fixable; Invitrogen), along with 200 pg of cas mRNA. To abolish the host endoderm specifically, we injected cas MO (1 ng; Open Biosystems) into hosts. After injections, donor and host embryos were dechorionated by treatment with pronase (0.1 mg/ml; Roche Molecular Biochemicals). Cell transplants were performed at mid-blastula stages. Two independent groups of cells were transplanted along the blastoderm margin of each host, ~90°~180° apart from each other, to increase the likelihood of contributing to the whole endoderm. For transplants using cas-overexpressing smo mutants as donors, we genotyped the donors right after transplantation. For transplants using smo mutants as hosts, we identified the mutant hosts by the somite phenotype (Chen et al., 2001) and genotyped with dissected tail and head tissues to confirm.

Purmorphamine Treatment

Embryos from Tg(ins:GFP):fh<sup>AV</sup>1 heterozygous increases were raised on agarose-coated plates after manual dechorionation. A 10 mM stock of purmorphamine (EMD Biosciences) was prepared in DMSO and diluted to 25 μM in 1% DMSO solution with egg water.

Histchemical Methods

Whole-mount in situ hybridization was performed as previously described (Alexander et al., 1998), using the following probes: pdx1 and insulin (Biemar et al., 2001).

The antibodies used are listed in the Supplemental Data. Embryos were fixed with 3% formaldehyde in 0.1 M Pipes, 1.0 mM MgSO<sub>4</sub>, 2 mM EGTA O.N. at 4°C. The yolk was manually removed, and embryos were blocked for 1 hr in PBS with 4% BSA and 0.3% Triton X-100. Primary and secondary antibodies were incubated O.N. For transverse sections, whole-mount embryos stained with antibodies were embedded in 4% low melting agarose and sectioned on a Leica VT1000S vibratome into 100 μm thick slices. The whole-mount embryos and sections were mounted in Vectashield (Vector Laboratories) and imaged with a Zeiss Lumar fluorescent stereomicroscope and LSM5 Pascal confocal microscope.

Lineage Tracing

Tg(sox17:GFP<sup>AV</sup>)<sup>AV</sup>70 embryos were injected with 3 nl of 2% photoactivatable DMNB-caged fluorescein dextran conjugate as a lineage tracer (10 kDa; Molecular Probes) in 0.2 M KCl and allowed to develop until the 6-somite stage (12 hpf). After dechorionation, embryos were mounted dorsally in the transplantation mold (Carmany-Rampey and Moens, 2006) filled with egg water. Using a Nikon C1si spectral confocal microscope, we visualized the endodermal sheet in live embryos at the 6- to 8-somite stage, and the A-P position of endodermal cells was determined by counting somites. Caged fluorescein was activated in two endodermal cells in each embryo with a 405 nm laser
focused through a 40× objective lens. The embryos were fixed at 14 or 50 hpf with 4% PFA and stained with antibodies against GFP, the uncaged fluorescein, and Insulin.

SUPPLEMENTAL DATA

Tables and additional figures of transplantation data, additional figures of phenotypic analyses, and Supplemental Experimental Procedures are included in the Supplemental Data available with this article online at http://www.developmentalcell.com/cgi/content/full/14/4/582/DC1/.

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