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p57 and Hes1 coordinate cell cycle exit with self-renewal of pancreatic progenitors

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Abstract

In developing organs, the regulation of cell proliferation and cell cycle exit is coordinated. How this coordination is achieved, however, is not clear. We show that the cyclin kinase inhibitor p57 regulates cell cycle exit of progenitors during the early stages of pancreas formation. In the absence of p57, the number of cycling progenitors increases, although expansion of progenitor population is prevented by apoptosis. We report that *p57* is a direct target of transcriptional repression by Notch effector, Hes1. Inactivation of *Hes1* results in the upregulation of p57 expression in progenitors, leading to cell cycle arrest, precocious differentiation and depletion of the progenitor pool. We present evidence that in *p57*/*Hes1* double null embryos, the absence of apoptosis results in the expansion of the progenitor population. We propose that Hes1 and p57 not only coordinate cell cycle exit and self-renewal of pancreatic progenitors during an early stage in organogenesis to regulate the number of pancreatic progenitors, but could also constitute a surveillance system to eliminate cells with aberrant cell cycle characteristics.

Keywords: Cell cycle; Pancreas; Notch; Organogenesis; Diabetes; Cancer; Proliferation; Apoptosis

Introduction

During organogenesis, proliferation of progenitor cells is coordinated with differentiation to ensure the correct size, shape and cellular make-up of the organ. The balance between self-renewal and programmed withdrawal of cells from the cell cycle is not only critical for the formation of appropriate number and types of differentiated cells, but also to prevent cells from acquiring aberrant proliferative characteristics that can lead to the initiation of cancer. The mechanisms that balance proliferation and cell cycle exit as well as perceive aberrant cell cycle behaviors are not yet well understood. During the formation of the pancreas, endocrine cell types are generated in a spatial temporally regulated fashion and direct lineage tracing analyses indicate that a population of progenitor cells persists to allow the differentiation of new endocrine cells throughout embryogenesis (Gu et al., 2002; Herrera, 2000). Thus, the development of the pancreas serves as a good model to study the molecular basis of how progenitor self-renewal and cell cycle exit are coordinated during organogenesis.

The pancreas originates during embryogenesis as evaginations from the posterior foregut endoderm to form epithelial buds (reviewed in Edlund, 1999; Hebrok et al., 2000; Wells and Melton, 1999). These epithelial buds consists of cells that express the homeogene, *Pdx1* (Ahlgren et al., 1996; Offield et al., 1996). Direct lineage tracing using the Cre/LoxP method demonstrated that the Pdx1-expressing cells are multipotent pancreatic progenitor cells and give rise to all mature pancreatic cell types (Gannon et al., 2000; Gu et al., 2003; Herrera et al., 2002). Several studies suggest that cell–cell signaling involving the canonical Notch pathway plays a crucial role in maintaining a population of progenitor cells. Targeted disruption of Notch ligand Delta-like 1(*Dll1*), intracellular mediators RBP-J_K, or *Hes1* resulted in precocious differentiation that led to a depletion of progenitor cells (Apelqvist et al., 1999; Jensen et al., 2000). The similarity in the phenotypes of mice genetically

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altered at several steps in the Notch signaling pathway indicated that canonical Notch signaling acting via *Hes1* regulates progenitor self-renewal in early pancreatic development. Further support for the role of Notch signaling in regulating differentiation and self-renewal in the pancreas comes from gain-of-function experiments in which Notch activation in the progenitor cells prevented differentiation of endocrine cells (Hald et al., 2003; Murtaugh et al., 2003). These results suggest that Notch-mediated signaling blocks the differentiation of pancreatic progenitor cells and instead forces the cell to remain in an undifferentiated state.

The decision to differentiate is made at the G_1-phase where D-type cyclins and cyclin kinase inhibitors (CKIs) play an important role in regulating cell cycle exit. CKIs regulate cell cycle progression by blocking phosphorylation of the retinoblastoma protein. Two groups of CKIs have been described (reviewed in Sherr and Roberts, 1999). These include the Ink4 family members that specifically inhibit cyclin D-Cdk4/6 activity, and the CIP/KIP family that includes $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, which exhibit promiscuous CDK-inhibitory activity. How does the G_1 cell cycle machinery interact with the Notch pathway to block differentiation and promote self-renewal? We show that *p57* mediates cell cycle exit of pancreatic progenitors and is a target of transcriptional repression by *Hes1*. Inactivation of Hes1 led to increased number of progenitors expressing p57 that precociously exited the cell cycle. This prompted us to ask whether the absence of *p57* would rescue the defects in the self-renewal of the progenitors in *Hes1* mutants. We show that in *p57*/*Hes1* double null embryos, self-renewal of pancreatic progenitors was rescued and led to an expansion of the pancreatic bud. These observations support a proposal that Hes1 and p57 coordinate the binary decision choice of pancreatic progenitor to either exit the cell cycle or self-renew.

Materials and methods

Animal husbandry and embryo dissection

Targeted disruption of the $p57^{Kip2}$ and *Hes1* alleles has been described previously (Zhang et al., 1997; Ishibashi et al., 1995). To ensure the maternal inheritance of the disrupted p57 allele, all matings were between a male $p57^{Kip2+/+}$ and $p57^{Kip2+/-}$ females in a C57BL/6 background. Embryos were isolated from these mice and considered to be 0.5 days of gestation at noon of the day the plugs were detected. Embryos were dissected in cold PBS, fixed in 4% formaldehyde for 45 min to 3 h, depending on the age of the embryo, followed by dehydration in ethanol and stored at −20°C until processed for paraffin embedding. Genomic DNA was extracted from the excess tissue using the DNeasy kit (Qiagen). Genotyping was performed as previously described (Caspary et al., 1999; Ohtsuka et al., 1999). Genotyping was also confirmed by performing immunohistochemistry (as described below) for $p57^{Kip2}$.

Immunohistochemistry

The gastrointestinal tracts were oriented during the embedding process in paraffin so that sections were cut along the anterior–posterior axis. Five-micrometer sections were deparaffinized in toluene, rehydrated in grades of alcohol and washed in H_2O. All slides were subject to antigen retrieval protocols using Antigen Unmasking Buffer (Vector Labs). After antigen unmasking, the slides were cooled to room temperature. All slides were permeabilized in 0.2% Triton X-100/TBS for 20 min, and non-specific binding of antibodies blocked with 0.2%Tween 20/3% IgG-free BSA/TBS. Primary antibodies were diluted in the blocking solution at the following dilutions: mouse anti-Glucagon 1:1000 (Sigma); rabbit anti-Glucagon 1:500 (Immunostar); guinea pig anti-insulin 1:500 (Dako); goat anti-p57 1:200 (clone M-20, Santa Cruz); rabbit anti-cleaved caspase-3 1:200 (Cell Signaling); mouse anti-Isl1 (39.4D5)1:50 (Developmental Studies Hybridoma Bank); rabbit anti-Hes1 1:100 (gift of T. Sudo); rabbit anti-phospho-histone H3 1:200 (Upstate); mouse anti-Pdx1 1:500 (a gift from J. Habener); rabbit anti Ngn3 1:200 (gift of M. Sander). Donkey- and goat-derived secondary antibodies conjugated to FITC, Cy3 or AMCA were diluted 1:500 (Jackson Laboratories). All slides were mounted with Vectashield with or without DAPI (Vector Labs). Slides were viewed using a Leica DM6000 microscope and images acquired using Openlab software.

BrdU injections, proliferative index and cell quantification

BrdU (25 μg per g of body weight) was injected intraperitoneally into pregnant dams 30 min (or otherwise stated) before harvesting the embryos. Mouse anti-BrdU antibody (1:100) (Amersham/Pharmacia) and the Pdx1 antibody were diluted in the nuclease buffer provided with the BrdU antibody. For calculation of the proliferative index, the number of Pdx1-positive cells and Pdx1- or BrdU-positive cells in the dorsal pancreatic buds were counted and the percentage of BrdU incorporation calculated (proliferative index). For cell quantification, four sections (3 sections apart) from each of three $p57^{+/-m}$ pancreas and three wild-type littermates were analyzed in this manner, giving a total of 24 data points. Quantification of progenitor population was carried out using three embryos from each genotype. Sections were stained for Pdx1 and Isl1 and five sections from each embryo were used to calculate the number of $Pdx1^+$ and $Isl1^+$ cells. The relative percentage of each population was calculated (for example, $Pdx1^+/Pdx1^+ + Isl1^+ \times 100$). Statistical significance was determined using Student's *t* test.

RNA isolation, RT-PCR and reporter gene assay

The pancreas were dissected in cold PBS and homogenized in TRI Reagent (Molecular Research Center) and total RNA prepared according to the manufacturer's methods (Chomczynski and Sacchi, 1987). RT-PCR was performed as described previously (Bhushan et al., 1998). cDNA was synthesized from RNA using a standard reverse transcription reaction. cDNA was amplified using PCR Mastermix (Qiagen) and the following thermal cycler protocol: 94° denaturation for 2 min, 35 cycles of 55° annealing for 1:30, 72° extension for 1 min, 93° denaturation for 1 min and a final extension at 72° for 5 min. Primers used were $p27^{Kip1}$: forward—cgactttcagaatcataagc, reverse—cttaattcggagctgtttac; $p57^{Kip2}$: forward—atctgacctcagacccaat, reverse—gccgttagcctctaaacta; $p21^{Cip1}$: forward—ggaactttgacttcgtcac, reverse—ctttaagtttggagactgg.

Cos7 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. The transfections were performed using Lipofactamine 2000 (Invitrogen) according to manufacture's instruction. CAT assay was carried out 24 h after the transfection using CAT ELISA kit (Roche). The level of the expression of CAT was normalized to beta-galactosidase activity expressed from a co-transfected vector.

Results

p57 is expressed in pancreatic progenitor cells that have exited the cell cycle

As Cip/Kip cell cycle regulators play an important role in coordinating cell cycle exit with terminal differentiation (reviewed in, Casaccia-Bonnefil and Liu, 2003), we analyzed which Cip/Kip family members were expressed in the early developing pancreas. RT-PCR on the pancreatic region of the developing gut showed that *p57* was strongly expressed at the early stages of pancreatic bud formation (Fig. 1A). We

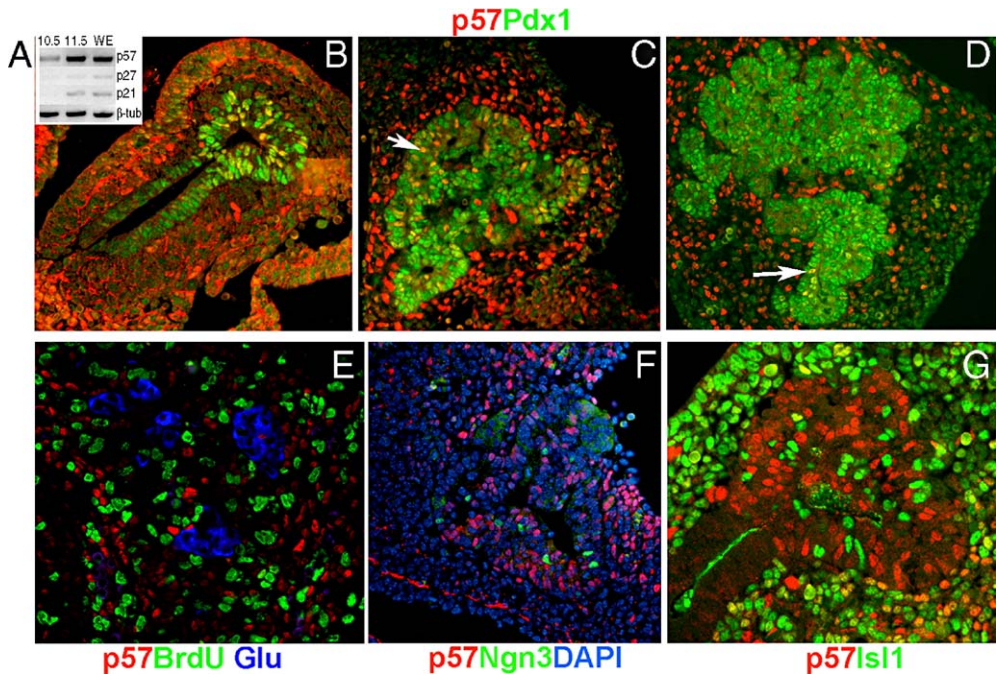

Fig. 1. p57 is expressed in pancreatic progenitor cells that have exited the cell cycle. RT-PCR expression analysis of *Cip/Kip* family in the pancreatic region of the developing embryo: *p57* was the predominant Cip/Kip family member expressed in the pancreatic bud (A). By immunofluorescence analysis, p57 was found to be expressed in the pancreatic epithelium for a short duration during early pancreatic development. At E10.5, p57 was expressed in a subset of Pdx1+ epithelial progenitor cells (B). A subset of epithelial progenitors continued to express p57 at E11.5 and strong expression was also observed in the pancreatic mesenchyme (C). By E12.5, very few epithelial progenitors expressed p57, although expression was still observed in the pancreatic mesenchyme (D). p57 was not expressed in cells that incorporated BrdU at E11.5 (E) p57 was not co-expressed with transcription factors that mark early differentiated endocrine cells at E11.5. p57 expression does not overlap with Ngn3 (F) or Isl1 (G).

examined the expression pattern of p57 by performing immunohistochemistry during embryogenesis. The expression of p57 was detected at embryonic day (E) 10.5, and co-staining with Pdx1 revealed that p57 expression was restricted to a subset of progenitor cells within the epithelial bud (Fig. 1B). At E11.5, p57 expression continued to be detected in a subset of progenitor cells (Fig. 1C) and strong expression was also detected in mesenchymal cells surrounding the epithelial bud. Expression of p57 at this stage was also detected in the progenitor cells of the ventral pancreatic buds (not shown). By E12.5, however, few pancreatic epithelial cells that expressed p57 were detected, although strong expression of p57 persisted in the pancreatic mesenchyme (Fig. 1D). p57 expression was not detected in the pancreatic epithelium at later stages of embryogenesis, indicating that p57 expression in pancreatic progenitors was limited to a short period of pancreatic organogenesis.

Next we examined the expression of p57 in progenitor cells in relation to cell cycle progression. Pregnant dams were injected with bromodeoxyuridine (BrdU) and embryos were analyzed for the expression of p57. No overlap of p57 expression was observed with BrdU after 1/2-hour pulse, which indicated that p57-expressing cells were not in S-phase (Fig. 1E). To correlate the expression of p57 with the generation of differentiated postmitotic cells during the formation of the pancreas, we stained the pancreatic bud with p57 and Ngn3 or Isl1, transcription factors that are early markers for endocrine precursor cells. (Ahlgren et al., 1997; Gradwohl et al., 2000). The majority of p57-expressing cells did not overlap with Ngn3 or Isl1 (Figs. 1F and G). These expression analyses indicated that p57 could mediate cell cycle exit of pancreatic progenitors but did not appear to play a role in differentiated endocrine cells.

Pancreatic progenitor cells reenter the cell cycle in the absence of p57

We reasoned that if p57 played a role in mediating cell cycle exit of pancreatic progenitors, the loss of *p57* would lead to an increase the number of cycling progenitors. To test this, we analyzed whether the proliferation of pancreatic progenitor cells was affected in mice in which the *p57* gene had been inactivated by homologous recombination in ES cells (Zhang et al., 1997). *p57* is imprinted such that only the maternal allele is active; therefore, offspring that inherit the inactivated allele of *p57* from $p57^{+/-}$ females are designated $p57^{+/-m}$ and are null for *p57* expression. To determine if the proportion of cycling pancreatic progenitor cells changed due to the absence of *p57*, embryos were pulse labeled with BrdU to mark cells in S-phase, then stained with Pdx1 and BrdU. In E11.5 wild-type embryos, around 25% of the pancreatic progenitor cells were in S-phase and tended to reside in the peripheral layer of the epithelial bud (Fig. 2A). The proportion of progenitor cells in S-phase was significantly increased in the pancreatic bud of $p57^{+/-m}$ littermate embryos (Fig. 2B). Quantification of S-phase index

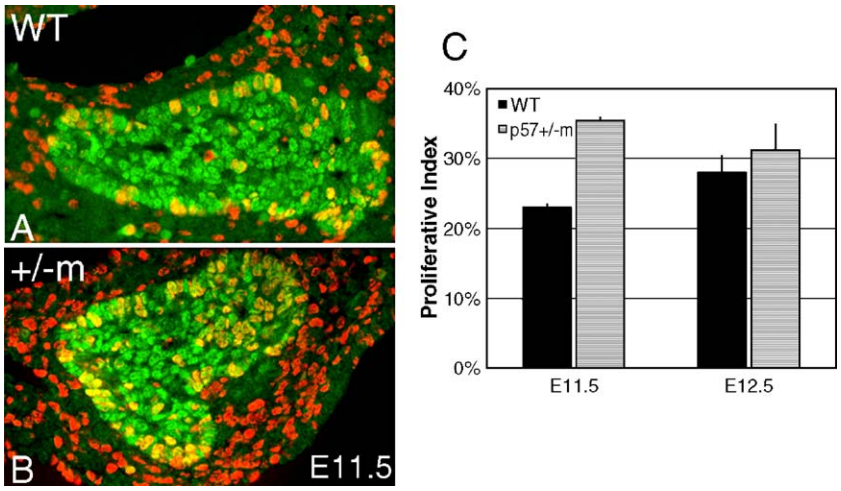

Fig. 2. Increased cycling progenitors at the expense of endocrine differentiation in $p57^{+/-m}$ pancreatic bud. The number of pancreatic progenitors that incorporate BrdU in wild-type embryos (A) was increased in $p57^{+/-m}$ littermates (B). Quantification of the proliferation index showed that at E11.5, more progenitor cells incorporated BrdU in $p57^{+/-m}$ pancreas (35.4 ± 0.4) compared to wild-type littermates (22.9 ± 0.5) $P < 0.001$ (C). However, by E12.5, the numbers of progenitors incorporating BrdU were similar in $p57^{+/-m}$ (28.2 ± 1.2) and wild-type littermates (31.0 ± 2.4) $P > 0.5$. The number of Pdx1-positive cells and Pdx1- or BrdU-positive cells in the dorsal pancreatic buds were counted and the percentage of BrdU incorporation calculated (proliferative index). For cell quantification, four sections (3 sections apart) from each of three $p57^{+/-m}$ pancreas and three wild-type littermates were analyzed in this manner, giving a total of 24 data points. Statistical significance was determined using Student's *t* test.

of progenitor cells indicated that an additional 15% of the progenitor cells in the pancreatic epithelium from *p57* null embryos were in S-phase entry compared to wild-type littermates; 22.9 ± 0.5 (WT) versus 35.4 ± 0.4 ($p57^{+/-m}$), $P < 0.001$ (Fig. 2C). This increased percentage of S-phase progenitor cells corresponded roughly to the percentage of progenitor cells that would have expressed p57 at this stage in development (Fig. 1B). However, one day later at E12.5, when p57 expression is barely detectable in the pancreatic epithelium but expressed strongly in the mesenchyme, the proportion of S-phase cells in the pancreas from $p57^{+/-m}$ and wild-type littermates embryos were similar 28.2 ± 1.2 (WT) versus 31.0 ± 2.4 ($p57^{+/-m}$) $P > 0.5$ (Fig. 2C). Thus, the loss of mesenchymal p57 expression did not appear to have any affect on the cell cycle characteristics of pancreatic progenitors. This suggests that p57-mediated cell cycle exit of pancreatic progenitors was not likely to be due to indirect effects of the mesenchyme on the pancreatic epithelium.

Reentry of progenitors into the cell cycle was compensated by apoptosis in $p57^{+/-m}$ embryos

The increased proliferation of pancreatic progenitor cells in $p57^{+/-m}$ embryos did not result in any obvious increase in the size of the pancreatic epithelium, which lead us to question whether these progenitor cells underwent apoptosis. The expression of activated caspase-3 was used as an indicator for initiation of apoptosis (reviewed in Nicholson, 1999). Apoptotic nuclei were rarely observed in the pancreatic epithelium from wild-type embryos at E10.5 (Fig. 3A). By contrast, widespread apoptosis was observed in the pancreatic epithelium in the $p57^{+/-m}$ littermate embryos (Fig. 3B). These cells had typical apoptotic morphology and were apoptotic by other assays such as TUNEL (data not shown). The activated caspase-3 staining within the epithelial bud did not overlap with Isl1, which indicated that the apoptosis observed in the pancreas was limited to progenitor cells, not derived from postmitotic endocrine cells. Apoptosis was also observed within the pancreatic epithelium of $p57^{+/-m}$ embryos at E11.5 (Figs. 3C and D) but very few apoptotic cells were observed within the pancreatic epithelium of $p57^{+/-m}$ embryos at E12.5 (not shown) consistent with the fact that p57 expression in pancreatic epithelium declines by E12.5. These results suggest that in the absence of *p57*, cell cycle reentry of progenitor cells that would otherwise have exited the cell cycle lead to apoptosis.

Hes1 suppresses p57 expression

Increased progenitor self-renewal at the expense of differentiation in $p57^{+/-m}$ developing pancreas was opposite of the phenotype observed in $Hes1^{-/-}$ embryos, in which precocious differentiation occurred at the expense of pancreatic progenitor self-renewal (Apelqvist et al., 1999; Jensen et al., 2000). We reasoned that precocious differentiation of pancreatic progenitor cells in mice that lack the *Hes1* would have been preceded by cell cycle arrest. Therefore, we investigated if Hes1 interacted with p57 to regulate the balance of proliferation and cell cycle exit of pancreatic progenitors. To investigate whether p57-mediated cell cycle exit could be responsible for the precocious differentiation observed in $Hes1^{-/-}$ pancreas, we compared the expression of p57 in pancreatic progenitor cells in $Hes1^{-/-}$ and wild-type littermates. Because precocious differentiation resulted in depletion of pancreatic progenitors by E10.5, we analyzed the expression of p57 at an earlier time point when Pdx1-expressing progenitor cells were still present in the pancreatic epithelium of $Hes1^{-/-}$ embryos. Pdx1-expressing cells outline the developing dorsal bud at E9.5 in wild-type (Fig.

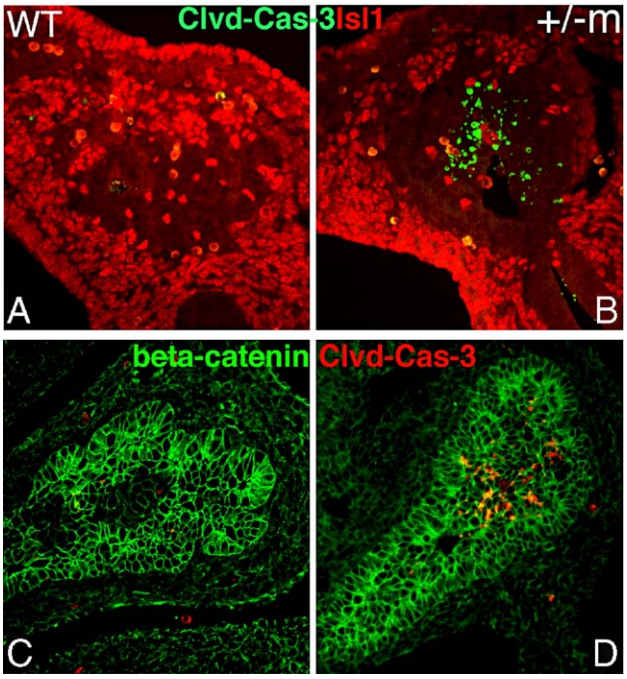

Fig. 3. Widespread apoptosis within the $p57^{+/-m}$ pancreatic epithelium. Using an antibody against the cleaved (activated) form of caspase-3, a marker for programmed cell death, very little apoptosis was observed in the wild-type pancreas at E10.5 (A). Widespread apoptotic cells were seen within the epithelial buds derived from $p57^{+/-m}$ littermates (B). Co-staining with Isl1 showed that pancreatic progenitors rather than differentiated endocrine cells underwent apoptosis. Co-staining of beta-catenin with the cleaved form of caspase-3 outlined the epithelial bud. Very few apoptotic cells were seen within the E11.5 pancreatic bud (C), although apoptotic cells were clearly seen within the epithelial bud of $p57^{+/-m}$ littermates (D).

4A) and $Hes1^{-/-}$ littermates (Fig. 4D). Co-staining with p57 revealed that, at this stage, very few Pdx1-expressing cells also express p57 in wild-type embryos (4.03 ± 2.09%) (Figs. 4B and C). By contrast, p57 was dramatically upregulated in the dorsal bud of $Hes1^{-/-}$ embryos (Fig. 4E) and most Pdx1-expressing cells within the dorsal bud also expressed p57 (74.01 ± 4.82%) (Fig. 4G). p57 expression was also observed in the progenitor cells of the ventral bud of $Hes1^{-/-}$ embryos (not shown). These results suggested that in the absence of *Hes1*, increased numbers of progenitors expressed p57 and shifted the progenitor fate to differentiation, thereby resulting in the depletion of progenitor cells.

The increased number of progenitors that expressed p57 in $Hes1^{-/-}$ pancreas led us to compare the expression pattern of Hes1 and p57 in the pancreatic bud of WT mice. Immunofluorescence analysis showed that p57 and Hes1 were expressed in distinct cells within the E10.5 pancreatic bud (Figs. 4H–J). p57-expressing cells were often adjacent to Hes1-expressing cells, but cells that expressed both Hes1 and p57 were not detected. The expression of Hes1 and p57 in distinct cells is likely to be a general phenomenon as Hes1 and p57 are also expressed in distinct cells in the neural tube (data not shown). To gain further insights into how p57 could interact with Hes1 in regulating cell cycle exit of pancreatic progenitors, we isolated the 7-kb sequence 5′ of the first exon of mouse *p57*. This 7-kb sequence contained two elements, *E-I* (214 bp) and *E-II* (199 bp) that are conserved in the human gene, suggesting that these elements could be involved in the regulation of *p57* expression. These two elements were utilized in a yeast one hybrid screen to identify if Herp/Hes family of transcriptional repressors interacted with the E-box CACCTG in *E-I* (P. Zhang, unpublished data). To test whether Hes1 could directly control the transcription of *p57*, we utilized a plasmid construct, *p57*-CAT, in which the 7-kb mouse *p57* promoter region was used to drive *CAT* (chlorophenicol acetyltransferase) reporter expression (Fig. 4K). Because the yeast hybrid screen suggested that the *E-1* element of the *p57* promoter region was responsible for the interaction with Hes1, we also constructed a plasmid $p57^{\Delta E\text{-}I}$-CAT, in which the *E-I* region was deleted (Fig. 4K). These CAT constructs were transfected to Cos7 cells alone or together with *Hes1* expression vectors. The CAT assay result (Fig. 4L) showed that co-transfection of *Hes1* led to suppression of CAT expression. Furthermore, the suppression of CAT was dependent on the *E-I* interacting region as co-transfection of Hes1 with $p57^{\Delta E\text{-}I}$-CAT failed to suppress CAT expression. Together, these results indicate that Hes1 can directly interact with the *p57* promoter and control proliferation through transcriptional repression of *p57*.

Rescue of pancreatic progenitors in p57/Hes1 double null embryos

Our results suggest that in $Hes1^{-/-}$ embryos, the upregulation of p57 in pancreatic progenitors resulted in cell cycle arrest of progenitor cells that led to the depletion of the progenitor population. As $p57^{+/-m}$ embryos showed the opposite phenotype, we investigated whether the loss of *p57* in the $Hes1^{-/-}$ embryos could rescue the proliferation of pancreatic progenitors. To test this, we generated *p57*/*Hes1* double null embryos. Based on mendelian ratios, fewer than expected *p57*/*Hes1* double null embryos were recovered at E11.5 and no double null embryos were recovered at later stages indicating embryonic lethality after E11.5. We analyzed the pancreas from E11.5 embryos by immunofluorescence staining for Pdx1 and glucagon to assess the pancreatic progenitor population and endocrine differentiation. As expected, the depletion of the progenitors at the expense of endocrine differentiation was clearly evident in the pancreatic region of the $Hes1^{-/-}$ embryos (Fig. 5B) compared to wild-type (Fig. 5A) and $p57^{+/-m}$ littermates (Fig. 5C). Significantly, however, a large number of pancreatic progenitors expressing Pdx1 were clearly seen in the pancreatic region of *p57*/*Hes1* double null embryos (Fig. 5D). The majority of the Pdx1-expressing progenitors of *p57*/*Hes1* double null embryos did not express Ngn3 indicating that these cells had not committed to an endocrine fate (data not shown). The pancreatic bud shown in Fig. 5D was representative of the expansion of $Pdx1^+$ cells seen in the double mutant embryos and quantification of the total number of $Pdx1^+$ cells from 5 sections per embryo from a total of three embryos per genotype confirmed that progenitor population was indeed expanded in *p57*/*Hes1* double null embryos compared to

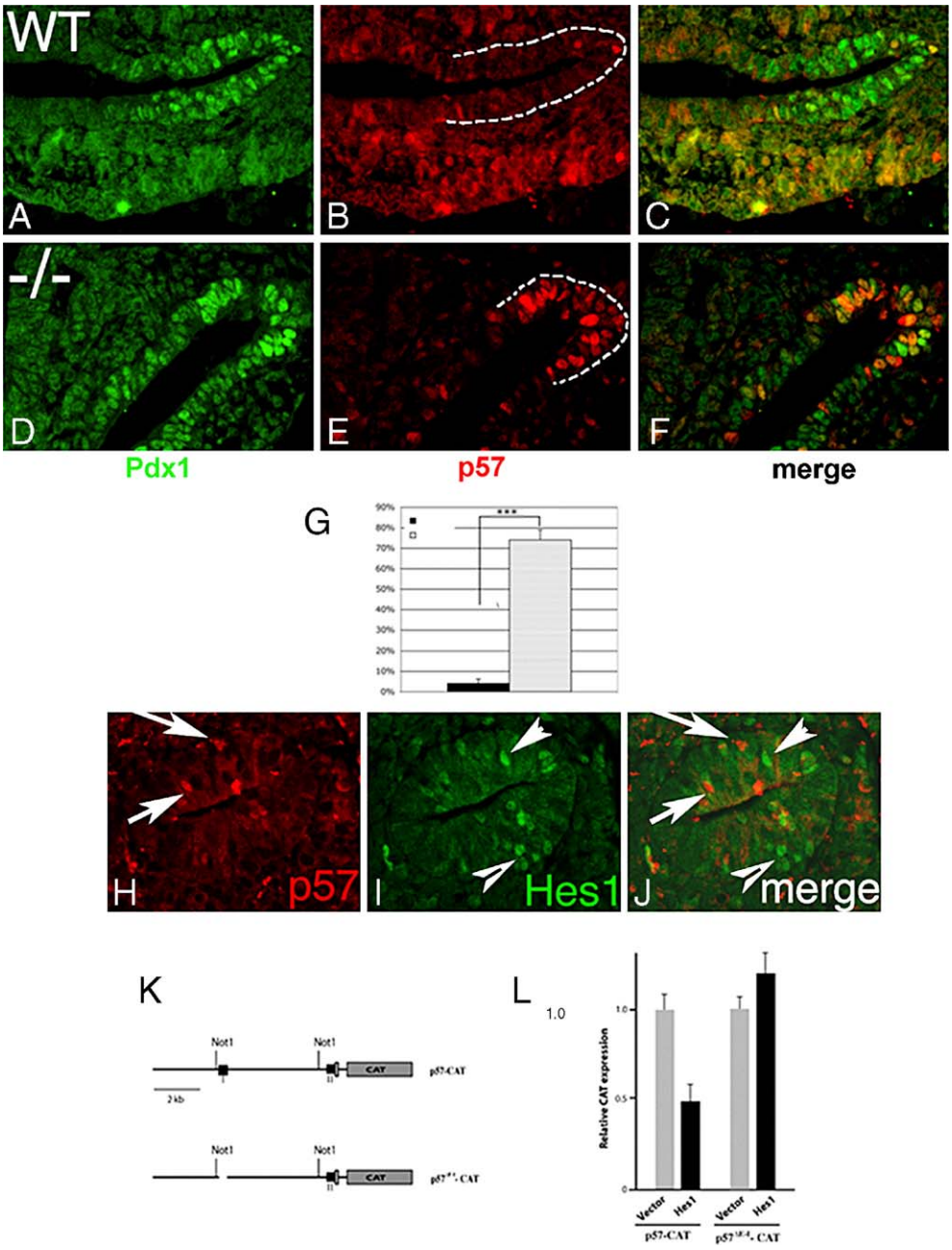

Fig. 4. p57 expression is suppressed by Hes1. (A–C) Wild-type embryos. At E9.5, Pdx1 expression marks the developing pancreatic bud (A). Very few cells within the p pancreatic bud (outlined) express p57 at this stage (B). Merged image showing p57-expressing epithelial cells co-express Pdx1 (C). (D–F) *Hes1*$^{-/-}$ littermate embryos. At E9.5, Pdx1-expressing progenitor cells are still present in the pancreatic bud, although depleted by E10.5 (D). In contrast to wild-type littermates, almost all the epithelial cells within the pancreatic bud (outlined) express p57 in *Hes1*$^{-/-}$ embryos (E). Merged images shows that these p57-expressing cells within the pancreatic bud co-express Pdx1 (F). Percentage of p57-expressing Pdx1^{+} cells in is greatly increased in *Hes1*$^{-/-}$ embryos when compared to WT littermates ($n = 3$) (74.01 ± 4.82% versus 4.03 ± 2.09%), $P < 0.001$ (G). Localization of p57 (H) and Hes1 (I) within the E10.5 pancreatic bud show that show that p57 and Hes1 are expressed in distinct cells (J). Constructs generated from the 7-kb mouse p57 promoter region used to drive CAT (chlorophenicol acetyltransferase) reporter expression included the two conserved elements E-I and E-II (K). Transfection of the CAT constructs into CV-1 cells together with Hes1 expression vector resulted in suppressed CAT expression that is E-I dependent (L).

wild-type littermates (Fig. 5E). To assess the balance of progenitor cells to differentiated endocrine cells, we calculated the relative percentage of Isl1^{+} cells and Pdx1^{+} cells to the total Isl1^{+} + Pdx1^{+} cells. Such analysis illustrated the near depletion of the progenitor population due to endocrine differentiation in *Hes1*$^{-/-}$ embryos and showed that loss of both *p57* and *Hes1* restored the balance of differentiation and progenitor population (Fig. 5F).

The expansion of the pancreatic bud led us to evaluate whether apoptosis of pancreatic progenitors was prevented in *p57*/*Hes1* double null embryos. We co-stained for the expression of glucagon to allow easy identification of the

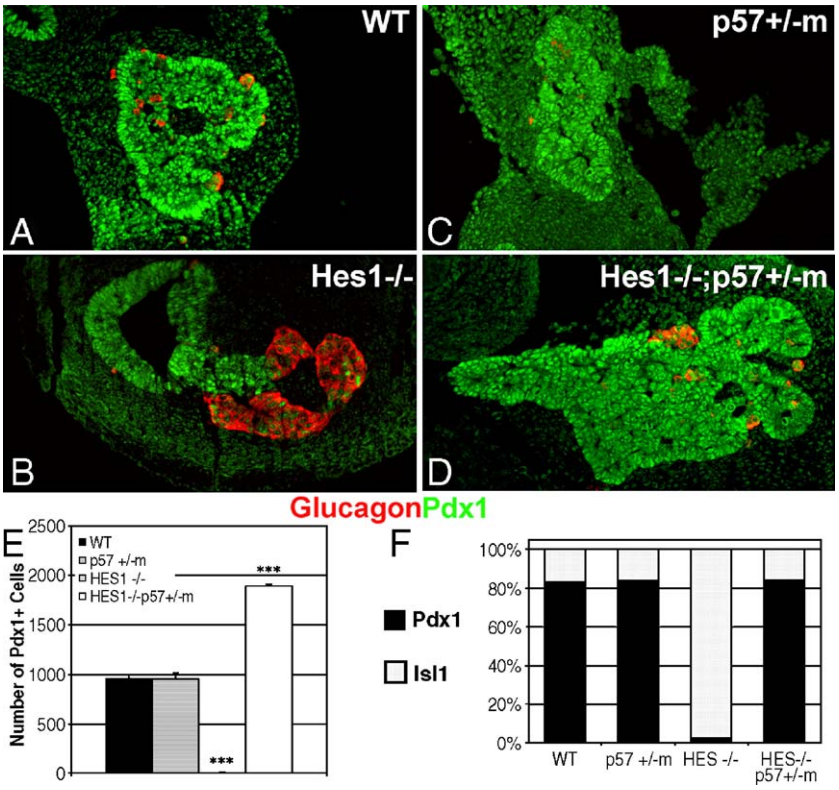

Fig. 5. Rescue of pancreatic progenitor pool in *p57*/*Hes1* double null embryos. To analyze the progenitor cell population, we stained the pancreatic region of E11.5 embryos for Pdx1 and glucagon (A). The depletion of the progenitors was evident in the pancreatic region of the $Hes1^{-/-}$ littermates (B). No increase in the progenitor cell population was evident in $p57^{+/-m}$ embryos (C); however, a dramatic expansion of the pancreatic progenitor population was evident in the *p57*/*Hes1* double null embryos (D). Quantification of the number of $Pdx1^{+}$ cells (5 section from 3 embryos of each genotype) showed expansion of the progenitor population in the *p57*/*Hes1* double null embryos (E). The proportion of $Isl1^{+}$ and $Pdx1^{+}$ cells in the pancreatic epithelium in the different genotypes as indicated and expressed as relative percentage of total $Isl1^{+}$ and $Pdx1^{+}$ cells. The depletion of progenitor cells in the $Hes1^{-/-}$ pancreatic epithelium due to precocious differentiation and rescue of the progenitor population in *p57*/*Hes1* double null embryos are evident (F).

pancreatic bud. Apoptotic pancreatic progenitors were rarely observed within the pancreatic buds derived from either wild-type (Fig. 6A) or *Hes1* homozygous littermates embryos (Fig. 6B) but was widespread in the pancreatic epithelium of $p57^{+/-m}$ embryos (Fig. 6C). Strikingly, the *p57*/*Hes1* double null embryos showed little evidence of apoptotic cells in the pancreatic epithelium (Fig. 6D). The absence of activated caspase-3 staining was not due to depletion of pancreatic epithelial cells, as nuclei staining showed a number of undifferentiated cells were present in the pancreatic bud (Fig. 6F). These results indicated that *Hes1* was involved in triggering the apoptosis of pancreatic progenitor cells in the $p57^{+/-m}$ embryos.

Discussion

During the early steps in the formation of the pancreas, the expansion of *Pdx1*-expressing multipotent progenitor population is coordinated with the differentiation of endocrine cells. Several studies have shown that Notch signaling via Hes1 plays a crucial patterning role in the development of the pancreas by selecting progenitor cells to either differentiate or self-renew (Apelqvist et al., 1999; Jensen et al., 2000; Murtaugh et al., 2003), although the mechanism by which Notch signaling regulates this binary decision process is unknown. Here we show that a mediator of cell cycle exit, *p57*, is a target of transcriptional repression by *Hes1*, a downstream target of Notch signaling and a known transcriptional repressor that has been shown to prevent differentiation by suppressing the expression of differentiation factors such as *Ngn3* (Lee et al., 2001). Thus, *Hes1* activation in pancreatic progenitors can suppress the expression of both *Ngn3* and *p57* to simultaneously prevent cell cycle exit and differentiation. Other studies indicate Hes type bHLH protein, *deadpan* has been shown to regulate *dacapo*, a CKI with homology to vertebrate p27 during the formation of optic lobes in *Drosophila* (Wallace et al., 2000). In addition, a recent study showed that *Hes1* was also capable of suppressing the expression of *p27* (Murata et al., 2005). Another study on oligodendrocyte specification in zebrafish showed that the p57 homolog is negatively regulated by Notch signaling (Park et al., 2005). Thus, the regulation of CKIs by the Hes family may be a conserved mechanism utilized to control proliferation during development.

We show that p57 is present during the early stages of pancreatic development (E10.5–E12.5) that correspond to massive expansion of the Pdx1-expresing progenitors. It is notable that p57 is not present during secondary transition (E13.5–E15.5) when the majority of endocrine cell differen-

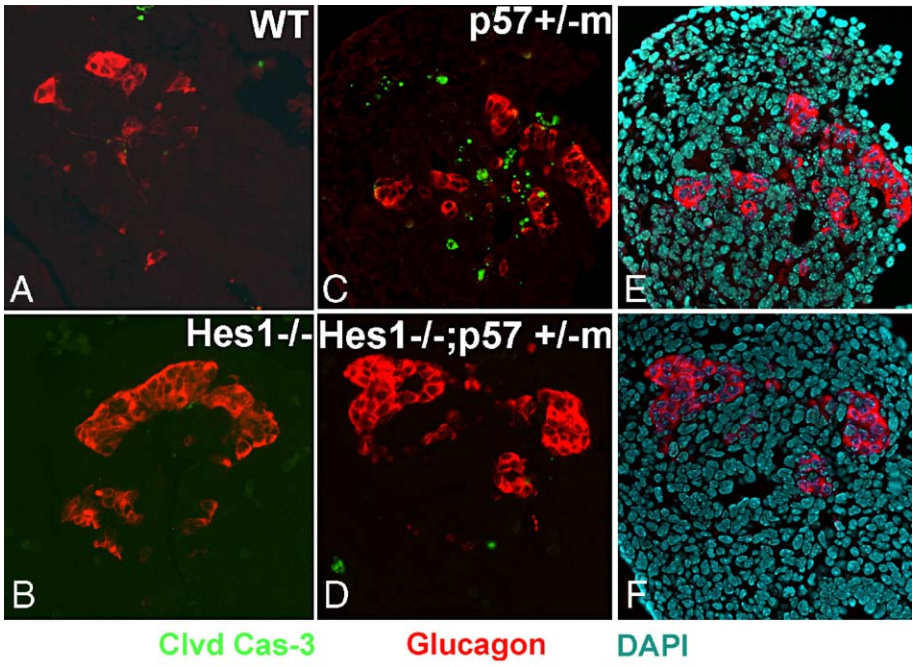

Fig. 6. Pancreatic progenitors in *p57*/*Hes1* double null embryos do not undergo apoptosis. We generated *p57*/*Hes1* double null embryos and analyzed pancreatic progenitors for apoptosis at E11.5 by immunofluorescence staining of activated caspase-3 and co-stained for the expression of glucagon that allowed easy identification of the pancreatic region. Apoptotic pancreatic progenitors were rarely observed within the pancreatic buds derived from either wild-type (A) or *Hes1* null littermates embryos (B) but widespread in the pancreatic epithelium of $p57^{+/-m}$ embryos (C). There was no evidence of apoptotic cells in the pancreatic epithelium of *p57*/*Hes1* double null embryos (D). (E, F) DAPI staining of the sections shown in panels C and D, respectively. The lack of apoptotic cells in panel D is not due to depletion of cells in the epithelial bud of *p57*/*Hes1* double null embryos (F).

tiation occurs, suggesting that other G_1 regulators are likely to be involved in regulating endocrine cell differentiation. Our results indicate the primary role of p57 during embryogenesis is to coordinate with Hes1 the balance between proliferation and cell cycle exit to regulate the number of pancreatic progenitors. The proliferation of multipotent progenitor cells must be precisely regulated as the different endocrine and exocrine cells types emerge at different times during development. For example, if progenitor cells exited the cell cycle too early, cell types that differentiate later in development will be reduced in the mature organ. Thus, properly controlled terminal cell cycle exit accompanied by differentiation early in organogenesis has repercussions for the size and the cellular make-up of the mature pancreas.

It is possible that suppression of p57 in progenitor cells allows them to respond to an extrinsic mitotic signal. We have previously shown that FGF10 is a mitotic signal expressed in the mesenchyme at E9.5–E11.5, the same time in organogenesis as the expression of p57 in epithelial progenitor cells (Bhushan et al., 2001). We propose that progenitors that express Hes1 and suppress the expression of p57 would respond to the extrinsic mitogen, FGF10, whereas progenitors that express p57 would disregard the FGF10 signal and exit the cell cycle. In support of this scenario, the activation of Notch targets by ectopic FGF10 expression as well as the requirements of the Notch signaling for the proliferative effects of FGF10 has been recently been shown (Hart et al., 2003; Miralles et al., 2006; Norgaard et al., 2003). It is worth pointing out that although *Hes1* is the major effector of the Notch pathway, Notch signaling on occasion can be effected by Hes1-independent events (reviewed in Louvi and Artavanis-Tsakonas, 2006). Furthermore, Hes1 expression alone does not mimic all of Notch's diverse effects on cells (Matsuno et al., 1997; Shawber et al., 1996). However, the similarities in the phenotypes of mice genetically altered at the level of ligand or downstream effectors indicated that Notch signaling acts via *Hes1* during early pancreatic development (Apelqvist et al., 1999; Jensen et al., 2000).

The reentry of pancreatic progenitors into the cell cycle, due to the absence of *p57*, is accompanied by widespread apoptosis. A similar phenomenon where inappropriately cycling cells are removed during organogenesis has been reported during retinal development. In retinal progenitors, p57 mediates cell cycle arrest and in *p57*-deficient retina, inappropriate S-phase reentry was quickly followed by apoptosis (Dyer and Cepko, 2000). Similarly, conditional deletions of *Rb* in the retina also result in inappropriate cell cycle activity and apoptosis (MacPherson et al., 2003, 2004). The results presented here indicate that Hes1, presumably acting downstream of Notch signaling, is required for the apoptosis of progenitors in the *p57*-deficient pancreas. As Hes1 and p57 are expressed in distinct progenitor cells in the pancreatic epithelium, non-cell autonomous mechanisms may be involved in the initiation of apoptosis in progenitors. Notch signaling is involved in establishing differences between adjacent cells and accumulating evidence indicates that reciprocal signaling involving feedback regulation amplifies and consolidates the differences between adjacent progenitors (Artavanis-Tsakonas et al., 1999). The cellular and developmental context of these adjacent progenitor cells dictates the

response to the reciprocal Notch signaling. We suggest that the cell cycle state is an important component of the cellular context of the progenitor cells perceived by Notch signaling. In this scenario, Notch signals to affect specific cell fate in a context-specific manner and inappropriately cycling cells that normally would have undergone cell cycle arrest instead undergo apoptosis. In an alternative scenario, it is possible that Hes1-expressing progenitors form a microenvironment from which survival factors are secreted. For example, Notch activation in the *Drosophila* wing can activate Wnt, a secreted morphogen and survival factor (Diaz-Benjumea and Cohen, 1995).

Uncontrolled proliferation of the progenitor cells due to the disruption of the coordination of self-renewal and differentiation could potentially set the stage for tumor formation. Increasing evidence is accumulating to support the hypothesis that disruption of Notch signaling and its downstream targets may play a role in certain cancers (Maillard and Pear, 2003). Notch downregulation appears to be critical during tumor progression in small cell lung cancer and in HPV-related cervical cancer (Talora et al., 2002). Furthermore, Notch acts as a tumor suppressor in the skin, as conditional inactivation of Notch1 in basal epidermal layers leads to spontaneous skin cancer (Nicolas et al., 2003). Elimination of inappropriately cycling cells may serve as a tumor suppressor function and the coordination between Hes1 and CKIs could be involved in perceiving and eliminating cells that have aberrant cell cycle states. Moreover, these findings may also be useful in the successful development of methods for ex vivo manipulation of pancreatic precursor cell proliferation and differentiation for therapeutic approaches to treat diabetes.

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References

Ahlgren, U., Jonsson, J., Edlund, H., 1996. The morphogenesis of the pancreatic mesenchyme is uncoupled from that of the pancreatic epithelium in IPF1/PDX1-deficient mice. Development 122, 1409–1416.

Ahlgren, U., Pfaff, S.L., Jessell, T.M., Edlund, T., Edlund, H., 1997. Independent requirement for ISL1 in formation of pancreatic mesenchyme and islet cells. Nature 385, 257–260.

Apelqvist, A., Li, H., Sommer, L., Beatus, P., Anderson, D.J., Honjo, T., Hrabe de Angelis, M., Lendahl, U., Edlund, H., 1999. Notch signalling controls pancreatic cell differentiation. Nature 400, 877–881.

Artavanis-Tsakonas, S., Rand, M.D., Lake, R.J., 1999. Notch signaling: cell fate control and signal integration in development. Science 284, 770–776.

Bhushan, A., Chen, Y., Vale, W., 1998. Smad7 inhibits mesoderm formation and promotes neural cell fate in *Xenopus* embryos. Dev. Biol. 200, 260–268.

Bhushan, A., Itoh, N., Kato, S., Thiery, J.P., Czernichow, P., Bellusci, S., Scharfmann, R., 2001. Fgf10 is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis. Development 128, 5109–5117.

Casaccia-Bonnefil, P., Liu, A., 2003. Relationship between cell cycle molecules and onset of oligodendrocyte differentiation. J. Neurosci. Res. 72, 1–11.

Caspary, T., Cleary, M.A., Perlman, E.J., Zhang, P., Elledge, S.J., Tilghman, S.M., 1999. Oppositely imprinted genes p57(Kip2) and igf2 interact in a mouse model for Beckwith–Wiedemann syndrome. Genes Dev. 13, 3115–3124.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Diaz-Benjumea, F.J., Cohen, S.M., 1995. Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal/ventral compartment boundary of the *Drosophila* wing. Development 121, 4215–4225.

Dyer, M.A., Cepko, C.L., 2000. p57(Kip2) regulates progenitor cell proliferation and amacrine interneuron development in the mouse retina. Development 127, 3593–3605.

Edlund, H., 1999. Pancreas: how to get there from the gut? Curr. Opin. Cell Biol. 11, 663–668.

Gannon, M., Herrera, P.L., Wright, C.V., 2000. Mosaic Cre-mediated recombination in pancreas using the pdx-1 enhancer/promoter. Genesis 26, 143–144.

Gradwohl, G., Dierich, A., LeMeur, M., Guillemot, F., 2000. neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proc. Natl. Acad. Sci. U. S. A. 97, 1607–1611.

Gu, G., Dubauskaite, J., Melton, D.A., 2002. Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development 129, 2447–2457.

Gu, G., Brown, J.R., Melton, D.A., 2003. Direct lineage tracing reveals the ontogeny of pancreatic cell fates during mouse embryogenesis. Mech. Dev. 120, 35–43.

Hald, J., Hjorth, J.P., German, M.S., Madsen, O.D., Serup, P., Jensen, J., 2003. Activated Notch1 prevents differentiation of pancreatic acinar cells and attenuate endocrine development. Dev. Biol. 260, 426–437.

Hart, A., Papadopoulou, S., Edlund, H., 2003. Fgf10 maintains notch activation, stimulates proliferation, and blocks differentiation of pancreatic epithelial cells. Dev. Dyn. 228, 185–193.

Hebrok, M., Kim, S.K., St Jacques, B., McMahon, A.P., Melton, D.A., 2000. Regulation of pancreas development by hedgehog signaling. Development 127, 4905–4913.

Herrera, P.L., 2000. Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. Development 127, 2317–2322.

Herrera, P.L., Nepote, V., Delacour, A., 2002. Pancreatic cell lineage analyses in mice. Endocrine 19, 267–278.

Ishibashi, M., Ang, S.L., Shiota, K., Nakanishi, S., Kageyama, R., Guillemot, F., 1995. Targeted disruption of mammalian hairy and enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. Genes Dev. 9, 3136–3148.

Jensen, J., Pedersen, E.E., Galante, P., Hald, J., Heller, R.S., Ishibashi, M., Kageyama, R., Guillemot, F., Serup, P., Madsen, O.D., 2000. Control of endodermal endocrine development by Hes-1. Nat. Genet. 24, 36–44.

Lee, J.C., Smith, S.B., Watada, H., Lin, J., Scheel, D., Wang, J., Mirmira, R.G., German, M.S., 2001. Regulation of the pancreatic pro-endocrine gene neurogenin3. Diabetes 50, 928–936.

Louvi, A., Artavanis-Tsakonas, S., 2006. Notch signalling in vertebrate neural development. Nat. Rev., Neurosci. 7, 93–102.

MacPherson, D., Sage, J., Crowley, D., Trumpp, A., Bronson, R.T., Jacks, T., 2003. Conditional mutation of Rb causes cell cycle defects without apoptosis in the central nervous system. Mol. Cell. Biol. 23, 1044–1053.

MacPherson, D., Sage, J., Kim, T., Ho, D., McLaughlin, M.E., Jacks, T., 2004. Cell type-specific effects of Rb deletion in the murine retina. Genes Dev. 18, 1681–1694.

Maillard, I., Pear, W.S., 2003. Notch and cancer: best to avoid the ups and downs. Cancer Cell 3, 203–205.

Matsuno, K., Go, M.J., Sun, X., Eastman, D.S., Artavanis-Tsakonas, S., 1997. Suppressor of hairless-independent events in Notch signaling imply novel pathway elements. Development 124, 4265–4273.

Miralles, F., Lamotte, L., Couton, D., Joshi, R.L., 2006. Interplay between FGF10 and Notch signalling is required for the self-renewal of pancreatic progenitors. Int. J. Dev. Biol. 50, 17–26.

Murata, K., Hattori, M., Hirai, N., Shinozuka, Y., Hirata, H., Kageyama, R., Sakai, T., Minato, N., 2005. Hes1 directly controls cell proliferation through the transcriptional repression of p27Kip1. Mol. Cell. Biol. 25, 4262–4271.

Murtaugh, L.C., Stanger, B.Z., Kwan, K.M., Melton, D.A., 2003. Notch signaling controls multiple steps of pancreatic differentiation. Proc. Natl. Acad. Sci. U. S. A. 100, 14920–14925.

Nicholson, D.W., 1999. Caspase structure, proteolytic substrates, and function during apoptotic cell death. Cell Death Differ. 6, 1028–1042.

Nicolas, M., Wolfer, A., Raj, K., Kummer, J.A., Mill, P., van Noort, M., Hui, C.C., Clevers, H., Dotto, G.P., Radtke, F., 2003. Notch1 functions as a tumor suppressor in mouse skin. Nat. Genet. 33, 416–421.

Norgaard, G.A., Jensen, J.N., Jensen, J., 2003. FGF10 signaling maintains the pancreatic progenitor cell state revealing a novel role of Notch in organ development. Dev. Biol. 264, 323–338.

Offield, M.F., Jetton, T.L., Labosky, P.A., Ray, M., Stein, R.W., Magnuson, M.A., Hogan, B.L., Wright, C.V., 1996. PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. Development 122, 983–995.

Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F., Kageyama, R., 1999. Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. EMBO J. 18, 2196–2207.

Park, H.C., Boyce, J., Shin, J., Appel, B., 2005. Oligodendrocyte specification in zebrafish requires notch-regulated cyclin-dependent kinase inhibitor function. J. Neurosci. 25, 6836–6844.

Shawber, C., Nofziger, D., Hsieh, J.J., Lindsell, C., Bogler, O., Hayward, D., Weinmaster, G., 1996. Notch signaling inhibits muscle cell differentiation through a CBF1-independent pathway. Development 122, 3765–3773.

Sherr, C.J., Roberts, J.M., 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev. 13, 1501–1512.

Talora, C., Sgroi, D.C., Crum, C.P., Dotto, G.P., 2002. Specific down-modulation of Notch1 signaling in cervical cancer cells is required for sustained HPV-E6/E7 expression and late steps of malignant transformation. Genes Dev. 16, 2252–2263.

Wallace, K., Liu, T.H., Vaessin, H., 2000. The pan-neural bHLH proteins DEADPAN and ASENSE regulate mitotic activity and cdk inhibitor dacapo expression in the *Drosophila* larval optic lobes. Genesis 26, 77–85.

Wells, J.M., Melton, D.A., 1999. Vertebrate endoderm development. Annu. Rev. Cell Dev. Biol. 15, 393–410.

Zhang, P., Liegeois, N.J., Wong, C., Finegold, M., Hou, H., Thompson, J.C., Silverman, A., Harper, J.W., DePinho, R.A., Elledge, S.J., 1997. Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith–Wiedemann syndrome. Nature 387, 151–158.