Notch inhibits Ptf1 function and acinar cell differentiation in developing mouse and zebrafish pancreas

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Summary

Notch signaling regulates cell fate decisions in a variety of adult and embryonic tissues, and represents a characteristic feature of exocrine pancreatic cancer. In developing mouse pancreas, targeted inactivation of Notch pathway components has defined a role for Notch in regulating early endocrine differentiation, but has been less informative with respect to a possible role for Notch in regulating subsequent exocrine differentiation events. Here, we show that activated Notch and Notch target genes actively repress completion of an acinar cell differentiation program in developing mouse and zebrafish pancreas. In developing mouse pancreas, the Notch target gene *Hes1* is co-expressed with *Ptf1-P48* in exocrine precursor cells, but not in differentiated amylase-positive acinar cells. Using lentiviral delivery systems to induce ectopic Notch pathway activation in explant cultures of E10.5 mouse dorsal pancreatic buds, we found that both Hes1 and Notch1-IC repress acinar cell differentiation, but not *Ptf1-P48* expression, in a cell-autonomous manner. Ectopic Notch activation also delays acinar cell differentiation in developing zebrafish pancreas. Further evidence of a role for endogenous Notch in regulating exocrine pancreatic differentiation was provided by examination of zebrafish embryos with homozygous *mindbomb* mutations, in which Notch signaling is disrupted. *mindbomb*-deficient embryos display accelerated differentiation of exocrine pancreas relative to wild-type clutchmate controls. A similar phenotype was induced by expression of a dominant-negative *Suppressor of Hairless* [*Su(H)*] construct, confirming that Notch actively represses acinar cell differentiation during zebrafish pancreatic development. Using transient transfection assays involving a Ptf1-responsive reporter gene, we further demonstrate that Notch and Notch/Su(H) target genes directly inhibit Ptf1 activity, independent of changes in expression of Ptf1 component proteins. These results define a normal inhibitory role for Notch in the regulation of exocrine pancreatic differentiation.

Supplemental data available online

Key words: Zebrafish, Mouse, Pancreatic, Exocrine, Embryo, p48

Introduction

During development, Notch signaling regulates cell fate decisions in a variety of epithelial and non-epithelial tissues. Following binding of cell-surface DSL (Delta, Serrate, Lag-2) ligands, Notch receptors undergo regulated intramembrane cleavage, resulting in liberation of the active intracellular (IC) domain (Mumm and Kopan, 2000). In the nucleus, liberated Notch-IC interacts with Suppressor of Hairless [Su(H); RBP-Jκ/CBF1 in mammals], resulting in transactivation of Notch/Su(H) target genes, including the Hairy Enhancer of Split (HES) family of bHLH transcriptional repressors (Beatus and Lendahl, 1998). HES family members generally act to maintain cells in an undifferentiated precursor state by further downregulating pro-differentiation factors such as *achaete* and *scute* in *Drosophila* proneurons (Fisher and Caudy, 1998), *MyoD* in mammalian myoblasts (Kuroda et al., 1999), and *Ngn3* in a neuroendocrine context (Lee et al., 2001). In a classical lateral inhibition paradigm, Notch prevents commitment to a primary or 'first available' cell fate, thereby reserving a population of undifferentiated precursor cells for ongoing proliferation and generation of later-appearing cell lineages (Fisher and Caudy, 1998). In more complex tissues, Notch signaling may be iteratively employed to regulate sequential cell fate decisions, allowing the orderly generation of multiple cell types from a common progenitor pool (Anderson et al., 2001; Posakony, 1994).

The mammalian pancreas is comprised of endocrine and exocrine lineages, both derived from a common progenitor pool

in foregut endoderm (Gu et al., 2003; Gu et al., 2002; Kawaguchi et al., 2002; Kim and MacDonald, 2002). Based on studies involving targeted inactivation of Notch pathway components in the mouse, it appears that Notch is required to prevent excessive precursor commitment to the endocrine lineage (Apelqvist et al., 1999; Jensen et al., 2000b). However, these gene targeting studies have been less informative with respect to a possible additional role for Notch in regulating exocrine lineage commitment. More recent studies have suggested that ectopic Notch activation may prevent acinar cell differentiation during murine pancreatic development (Hald et al., 2003; Murtaugh et al., 2003), and also induce dedifferentiation of exocrine cell types in adult pancreatic epithelium (Miyamoto et al., 2003). However, it remains to be determined whether these observations reflect a similar role for endogenous Notch pathway activation during development, and the mechanism for this effect remains unknown. In the current study, we find that Notch signaling is indeed active within a committed exocrine progenitor pool in developing mouse pancreas, and further demonstrate that Notch blocks terminal acinar cell differentiation, but not initial commitment to the exocrine lineage. In addition, we take advantage of altered lineage relationships in developing zebrafish pancreas to demonstrate a biologic role for endogenous Notch in developing exocrine pancreas.

Materials and methods

Immunofluorescent staining, confocal imaging and 3D image deconvolution

For immunolabeling, tissues were fixed, sectioned and stained as previously described (Esni et al., 2004). The following antibodies were used at the indicated dilutions for immunofluorescence analysis; rat monoclonal anti-E-cadherin (Zymed Laboratories, 1:200), goat polyclonal anti-vimentin (Santa Cruz Biotechnology, 1:50), rabbit polyclonal anti-p48 (gift from Helena Edlund, 1:1000), mouse monoclonal anti-nestin (Transduction Laboratories, 1:50), rabbit polyclonal anti-Pdx1 (gift from Christopher Wright, 1:500), goat polyclonal anti-amylase (Santa Cruz Biotechnology, 1:500), and guinea pig polyclonal anti-insulin and anti-glucagon (Linco Research, 1:1000). Two different anti-Hes1 antibodies were used: goat polyclonal anti-Hes1 (Santa Cruz Biotechnology, 1:75) and rabbit polyclonal anti-Hes1 (gift from Tetsuo Sudo, 1:50). The following reagents were purchased from Jackson ImmunoResearch Laboratories: Biotin-conjugated anti-rat (1:500), anti-guinea pig (1:500) and anti-goat IgG (1:500). Cy3- and Cy2-conjugated streptavidin (1:1000, 1:300), Cy3-conjugated anti-rabbit (1:300), and Cy5-conjugated anti-rat (1:300). For each antibody, specificity was confirmed using negative control slides omitting primary antibody. In the case of Hes1, specificity was further confirmed by preincubation of antibody with synthetic Hes1 peptide. Fluorescence confocal microscopy was performed with a confocal laser scanning microscope (410LSM, Zeiss) using a 40× (NA 1.5) C-apochromat objective, and image analysis was performed using MetaMorph series 5.0 software (Universal Imaging). For deconvolution microscopy, digital Z-stack images of labeled specimens were obtained at 1 μm intervals using an inverted motorized fluorescent microscope (Axiovert 200M, Zeiss), and image deconvolution was performed by Zeiss Axiovision 4D software using a regularized inverse filter algorithm.

Double in situ hybridization on tissue sections

For double fluorescent in situ hybridizations, embryonic mouse pancreas was fixed in 4% PFA and embedded in OCT. Sections were hybridized simultaneously with fluorescein-labeled *Hes1* and digoxigenin-labeled *Ngn3* riboprobes. Hybridized probes were sequentially detected using AP-conjugated anti-fluorescein antibody visualized with Fast Red (Roche), followed by a 30-minute post-fixation in 4% PFA and inactivation of anti-fluorescein-AP by acid quenching with 100 mM Glycine-HCl pH 2.2, 0.1% Tween-20. Following additional washes, sections were incubated with AP-conjugated anti-digoxigenin antibody, which was then visualized using ELF®97 In Situ Hybridization Kits (Molecular Probes). Probes against *Hes1* and *Ngn3* were kindly provided by Mahendra Rao (National Institute on Aging) and Douglas Melton (Harvard University).

Construction of dual promoter lentiviral vectors and lentivirus production

cDNAs encoding mHes1 (gift from Mary Hatten, Rockefeller University) (Solecki et al., 2001) and hNotch1-ICD (gift from Warren Pear, University of Pennsylvania) were cloned into the EF.v-CMV.GFP lentiviral vector (Yu et al., 2003) and sequence verified. Lentivirus production and concentration was performed as previously described (Yu et al., 2003). Briefly, VSV.G-pseudotyped recombinant lentiviruses were produced by transient transfection of the transducing vector into 293T cells, along with two packaging vectors: pMD.G, a VSV.G envelope-expressing plasmid, and pCMVΔR8.91, containing the HIV-1 *gag*/*pol*, *tat* and *rev* genes (1.5 μg: 2.0 μg: 0.5 μg ratio of these three vectors). Viral supernatants were collected at 24, 48 and 72 hours after transfection, and concentrated using filtration columns (Centricon Plus-20, molecular weight cutoff=100 kD; Millipore, Bedford, MA, USA). Additional lentiviral vector validation was accomplished by transduction of COS7 cells, with Hes1 protein expression confirmed by immunofluorescence and Notch1-ICD protein expression confirmed by both immunofluorescence and by activation of a Notch/Su(H)-responsive luciferase reporter (see Fig. S1 at http://dev.biologists.org/supplemental).

Explant culture of embryonic pancreas and lentiviral transgene gene delivery

Isolation, infection and culture of pancreatic rudiments were performed as previously described (Ahlgren et al., 1996; Esni et al., 2004). Prior to infection, intact dorsal pancreatic buds were dissected and incubated for 1 minute at room temperature in 0.6 U/ml dispaseI (Roche). After separating the epithelium from the surrounding mesenchyme, naked buds were submerged in 200 μl culture medium (BioWhittaker Medium 199, 10% fetal calf serum, 50 U/ml penicillin G-streptomycin, 1.25 μg/ml fungizone) and infected with appropriate lentiviral vectors overnight at 37°C in the presence of 8 μg/ml polybrene. For each E10.5 dorsal epithelial bud, 1×10^6 transfection units were utilized. Infected buds were then transferred onto Millicell-CM 0.4 μm inserts, recombined with E11.5 dorsal mesenchyme and cultured for six additional days. In the course of five independent experiments, a total of 20 dorsal bud explants were successfully infected, recombined with mesenchyme, and cultured (n=7 for GFP alone, n=6 for GFP;NotchIC, n=7 for GFP;Hes1). Following harvest, cryosections were prepared and stained for E-cadherin in combination with either insulin (examined on sections of five buds infected with GFP alone, four buds infected with GFP;NotchIC, and five buds infected with GFP;Hes1), glucagon (examined on sections of five buds infected with GFP alone, four buds infected with GFP;NotchIC, and five buds infected with GFP;Hes1), amylase (examined on sections of seven buds infected with GFP alone, six buds infected with GFP;NotchIC, and seven buds infected with GFP;Hes1), Ptf1-p48 (examined on sections of three buds infected with GFP alone, three buds infected with GFP;NotchIC, and three buds infected with GFP;Hes1), or nestin (examined on sections of three buds infected with GFP alone, three buds infected with GFP;NotchIC, and three buds infected with GFP;Hes1). To determine cell-autonomous effects of Notch1-IC and Hes1, the number of E-cadherin-positive epithelial cells expressing GFP in combination with insulin, glucagon, amylase, Ptf1-p48 or nestin was determined by direct counting of multiple bud

sections, and expressed as a fraction of the total number of E-cadherin/GFP-expressing cells. Two-sample Z-tests comparing GFP versus GFP:Notch1-IC and GFP versus GFP;Hes1 were performed using Stata 8.0 software (StataCorp LP).

Zebrafish stocks and embryo care

Wild-type (Scientific Hatcheries), *mib*^{ta52b} (Itoh et al., 2003), *hsp70:Gal4*, and *UAS:notch1a-ICD* (Park and Appel, 2003; Scheer et al., 2001) zebrafish strains were raised according to standard protocols (Westerfield, 2000). Embryos were raised at 28.5°C in E3 with 0.1 ppm methylene blue until 24 hpf, at which point they were transferred to E3 with 0.003% phenylthiourea (2 mM) to inhibit pigmentation and facilitate whole-mount examination. For Notch gain-of-function analyses, homozygous *hsp70:Gal4* females were mated to *UAS:notch1aICD* heterozygote males. *Notch1aICD* overexpression was induced by transferring embryos to 40°C media for 30 minutes. After in situ hybridization and phenotypic classification, embryos were genotyped by proteinase K digestion and subsequent PCR for the *UAS:notch1aICD* transgene using the following primers: 5′-CATCGCGTCTCAGCCTCAC and 5′-CGGAATCGTTTATT-GGTGTCG.

Capped mRNA injection

Full-length, capped *Su(H)* dominant-negative messenger RNA was generated by applying the SP6 mMessage kit (Ambion) to MluI-linearized pCSGSuDN (Lawson et al., 2001), which encodes a fusion protein comprised of EGFP and a dominant-negative DNA binding mutant of *Xenopus Su(H)*. The EGFP coding region from pEGFP-1 (Clontech) was subcloned into the pCDNA3 expression vector (Invitrogen) to enable generation of control full-length, capped EGFP mRNA (T7 mMessage, Ambion). Both mRNAs were diluted to a final working concentration of 100 ng/μL in Danieau's buffer (Westerfield, 2000). For injection, single-cell stage embryos were transferred to a molded agarose injection dish, and approximately 200 pg of mRNA was microinjected into the yolk of each embryo. For both constructs, expression was confirmed by GFP fluorescence beginning at 4-6 hpf and persisting until at least 32 hpf.

Analysis of zebrafish embryos by whole-mount in situ hybridization

Whole-mount in situ hybridization of *Danio rerio* embryos was performed as previously described (Lin et al., 2004). *Trypsin*, *pdx1* and *ptf1a-p48* probes were generated by PCR from adult zebrafish poly-A primed cDNA. Probes for *hhex* and *GATA6* have previously been described (Wallace and Pack, 2003), while probes for *insulin* and *glucagon* were provided by Victoria Prince (University of Chicago). Embryos were scored for the presence or absence of *ptf1a-p48* and *trypsin* expression by whole-mount observation in 75% glycerol. A test for independent proportions was performed to determine the statistical significance of differences in expression between control and experimental groups.

Plasmid and adenoviral constructs

Myc epitope-tagged human Notch1-IC and Notch1-IC truncation mutants were generated by PCR amplification of appropriate fragments followed by in-frame assembly in pCMV-Tag1 (Stratagene). All constructs were sequence verified and protein expression confirmed by western blot using anti-myc antisera (Santa Cruz). A myc-tagged Hes1 cDNA was obtained from Mahendra Rao (National Institute on Aging). Expression vectors encoding flag-tagged Hey1 and Hey2 were obtained from Larry Kedes (University of Southern California). An expression vector encoding human E47 was obtained from Michael Chin (Harvard University). Previously characterized Ptf1-responsive and Su(H)-responsive luciferase reporter vectors were obtained from from Masashi Kawaichi (Nara Institute) and Diane Hayward (Johns Hopkins University), respectively. The Ptf1-responsive luciferase reporter (Ptf1-luc) contains four tandem repeats of the Ptf1 binding site from the rat chymotrypsinogen reporter (Obata et al., 2001), whereas the Su(H)-responsive luciferase reporter [Su(H)-luc] contains eight tandem repeats of the Su(H) binding element (Hsieh et al., 1996). The construction and characterization of adenoviral vectors encoding GFP alone or in combination with either Notch1-IC, Hes1, Hey1 or Hey2 has been described previously (Miyamoto et al., 2003; Sriuranpong et al., 2001).

Cell culture, adenoviral infection and transient transfection

The AR42J rat acinar carcinoma line was cultured in F12 media with 10% FBS and infected with indicated adenoviral vectors at an MOI of 20:1. COS7 cells were cultured in modified DMEM medium with 10% FBS. Transfection was performed in 24-well plates at 90% confluence using Lipofectamine (GIBCO), according to manufacturer's protocol.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were isolated from AR42J cells using a modification of the procedure described by Dignam et al. (Dignam et al., 1983). EMSA was performed as previously described, using a ^{32}P-labeled, 21-bp double stranded oligonucleotide (sense strand, 5′-GTCACCTGTGCTTTTCCCTGC-3′) spanning the A element of the rat *elastase1* enhancer (Rose et al., 2001). For supershift, 1 μl of Ptf1-p48 antiserum was preincubated with the nuclear extract prior to oligonucleotide addition.

Luciferase assays

COS7 cells were transfected in 24-well plates with 0.5 μg of either the Ptf1-luc or the Su(H)-luc firefly luciferase reporter plasmids in combination with indicated expression vectors. In addition, 0.025 μg of the pRL-TK Renilla luciferase reporter plasmid (Promega) was added to control for transfection efficiency. Total DNA per transfection was kept constant at 1 μg per well by adding vector DNA as needed. After 24 hours, cells were harvested using the Promega Dual-Luciferase Reporter Assay System. For each condition, sequential measurement of firefly and Renilla luciferase was performed, and normalized luciferase activity reported as the ratio of firefly to Renilla luciferase activity. The reported data represent the mean results from three different experiments, each performed in triplicate. In addition, parallel samples were processed for western blotting using the following antibodies and dilutions: rabbit anti-rat Ptf1-P48 (Adell et al., 2000), 1:1000; rabbit anti-E47 (Santa Cruz), 1:1000; mouse monoclonal anti-myc (Invitrogen), 1:5000; goat anti-Notch1-IC (Santa Cruz C20), 1:1000; mouse monoclonal anti-flag (Sigma) 1:1000.

Results

Notch pathway activation in exocrine pancreatic precursors

In order to determine whether Notch pathway activation is a characteristic feature of exocrine pancreatic precursors, we assessed expression of the Notch/Su(H) target gene, *Hes1*, in conjunction with endocrine and exocrine lineage markers in developing mouse pancreas, using both double in situ hybridization and immunofluorescent confocal analysis. On E13.5, developing mouse pancreas is characterized by a branched epithelial tree comprised of largely uniform Pdx1-positive cells, lacking obvious morphologic features of exocrine differentiation. At this stage, dedicated endocrine precursors are marked by expression of *Ngn3*, whereas exocrine precursors are marked by expression of the helix-loop-helix transcription factor *Ptf1-P48* (Chiang and Melton,

2003). As demonstrated in Fig. 1, nuclear Hes1 protein is broadly detected within the nuclei of E13.5 Pdx1-positive epithelial cells, as well as in Pdx1-negative mesenchymal elements (Fig. 1A,D,G). Nuclear Hes1 protein is also detected in the majority of cells expressing Ptf1-p48, suggesting Notch pathway activation in an exocrine progenitor pool (Fig. 1B,E,H). In contrast, *Hes1* transcripts are not observed in *Ngn3*-positive epithelial cells, as demonstrated by double in situ hybridization (Fig. 1C,F,I), suggesting downregulation of Notch signaling in committed endocrine progenitors.

By E14.5, cells in the periphery of the branching epithelial tree begin to adopt a pyramidal shape and organize into discrete clusters, representing nascent acinar structures. At this time, nuclear Hes1 immunoreactivity remains evident in central E-cadherin-positive undifferentiated epithelium (Fig. 1J), as well as in a population of E-cadherin-negative mesenchymal cells. The mesenchymal identity of E-cadherin-negative, Hes1-positive elements was additionally confirmed by vimentin labeling (see Fig. S2 at http://dev.biologists.org/supplemental). Within the epithelium, Hes1 protein expression extends peripherally to include cells comprising epithelial branches, up to and including cells immediately adjacent to developing acini (‘centroacinar cells’; see arrows in Fig. 1J,K). Notably, Hes1 protein is absent in neighboring amylase-positive acinar cells (Fig. 1L). This abrupt transition from Hes1-positive, amylase-negative, undifferentiated epithelium to Hes1-negative, amylase-positive, acinar epithelium suggests a possible inhibitory role for Notch in the regulation of exocrine differentiation in developing pancreas.

Notch inhibits both endocrine and exocrine differentiation in developing mouse pancreas

In order to more directly evaluate the ability of Notch to regulate exocrine differentiation in developing pancreas, we determined the effects of forced Notch pathway activation in explant cultures of E10.5 dorsal pancreatic buds. Using replication-incompetent lentiviral vectors encoding either GFP alone, GFP plus the activated intracellular domain of murine Notch1 (GFP;NotchIC), or GFP plus murine Hes1 (GFP;Hes1), we effectively created mosaic epithelial buds, in which a subset of cells undergo forced Notch pathway activation. Infected buds were then recombined with dorsal mesenchyme and allowed to differentiate in vitro. As demonstrated in Fig. 2, developing pancreatic epithelial cells expressing GFP alone were fully capable of completing either endocrine or exocrine differentiation programs following six days of explant culture, as determined by immunofluorescent detection of insulin, glucagon, amylase and Ptf1-p48 in GFP-positive cells (Fig. 2D,G,J,M). In contrast, cells infected with either GFP;Hes1 or GFP;NotchIC were unable to differentiate into insulin-producing beta-cells, but were able to differentiate into glucagon-producing alpha cells (Fig. 2E,F,H,I). In order to quantify this effect, harvested epithelial buds were stained for E-cadherin to allow identification of GFP-expressing

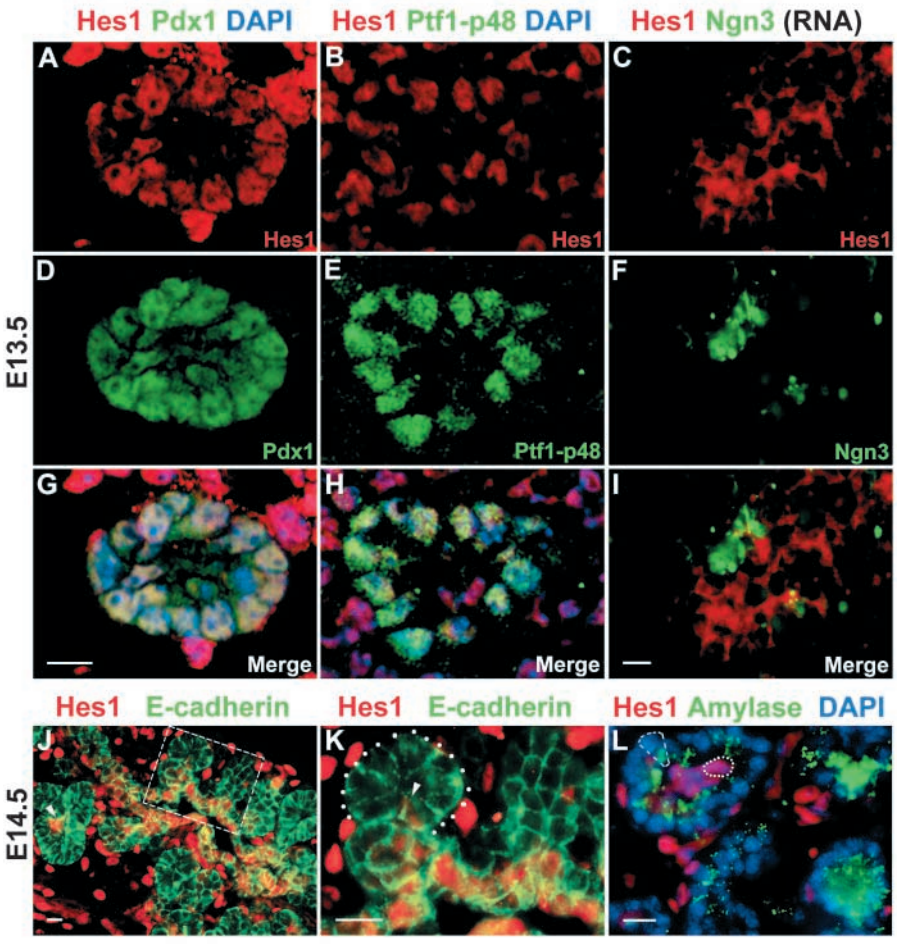


Fig. 1. Notch pathway activation assessed by Hes1 expression in developing mouse pancreas. (A,D,G) Single channel and merged confocal images demonstrating co-expression of Hes1 (red) and Pdx1 protein (green) in E13.5 mouse pancreas. (B,E,H) Single channel and merged confocal images demonstrating co-expression of Hes1 (red) and Ptf1-p48 protein (green) in E13.5 mouse pancreas. (C,F,I) Double fluorescent in situ hybridization demonstrating absence of *Hes1* expression in *Ngn3*-positive endocrine precursors. Cells with cytoplasmic *Hes1* transcripts (red) do not contain transcripts for *Ngn3* (green). (J) Immunostaining for Hes1 and E-cadherin on E14.5 pancreas reveals nuclear Hes1 protein in central undifferentiated epithelium as well as in epithelial branches and centroacinar cells (arrow). (K) Magnified view of area outlined in J, demonstrating nuclear Hes1 protein in cells comprising peripheral epithelial branches (arrow), but exclusion of Hes1 from acinar cluster, indicated by broken line. (L) Immunostaining for Hes1 and amylase, demonstrating Hes1 protein in nuclei of amylase-negative terminal ductal/centroacinar cells, but not in differentiating amylase-positive acinar cells. Dashed lines indicate contour of representative Hes1-negative acinar cell, identified by basal nuclei and apical collection of amylase-positive zymogen granules. Dotted lines indicate contour of representative Hes1-positive, amylase-negative terminal ductal/centroacinar cell. Images in C,F,I and J-L generated by 3D image deconvolution. Scale bars: 10 μm.

epithelial cells, and the number of cells expressing GFP in combination with markers of endocrine and exocrine differentiation was determined by direct counting (Fig. 3). Following infection with GFP alone, approximately 9% of GFP-positive epithelial cells were insulin-positive, compared with <1% following infection with either GFP;Hes1 or GFP;NotchIC.

With respect to the effects of ectopic Notch activation on exocrine lineage commitment and acinar cell differentiation, cells infected with either GFP;Hes1 or GFP;NotchIC were much less frequently able to complete an acinar differentiation program compared with cells infected with GFP alone, even while immediately adjacent uninfected cells were readily able to undergo acinar cell differentiation (Fig. 2J-L). Following infection with GFP alone, approximately half of GFP-positive epithelial cells underwent acinar cell differentiation marked by amylase (Fig. 3). The frequency of acinar cell differentiation among GFP-positive epithelial cells was reduced to 8-9% in cells infected with either GFP;Hes1 or GFP;NotchIC. In contrast, forced Notch activation had only a minor effect on the frequency of Ptf1-p48 expression among infected cells (Fig. 2M-O, Fig. 3), consistent with observed co-expression of Ptf1-p48 and Hes1 during normal development. In addition to Ptf1-p48, exocrine precursor cells have been demonstrated to express nestin, an intermediate filament also expressed in undifferentiated neural precursors (Delacour et al., 2004; Esni et al., 2004). Cells infected with either GFP alone or GFP;NotchIC continued to express nestin at normal frequencies, whereas a significant increase in the frequency of nestin expression was observed among epithelial cells infected with GFP;Hes1 (Fig. 3).

Based on these observations, we next examined co-expression of Ptf1-p48 and amylase following infection with either GFP alone, GFP;Hes1 or GFP;NotchIC. In control explants, the vast majority of Ptf1-p48-positive cells completed an acinar cell differentiation program, marked by expression of amylase. In contrast, forced Notch pathway activation resulted in the abnormal accumulation of a Ptf1-p48-positive, amylase-negative population (Fig. 4). Following infection with GFP alone, 83% of all infected Ptf1-p48-positive cells demonstrated positive staining for amylase at the time of explant harvest, compared with 18% and 22% in explants infected with GFP;Hes1 or GFP;NotchIC, respectively. These results were especially striking, with mosaic acini frequently demonstrating inclusion of GFP-positive, Ptf1-p48-positive, amylase-negative cells immediately adjacent to GFP-negative, Ptf1-p48-positive, amylase-positive cells following infection with either GFP;NotchIC or GFP;Hes1 (Fig. 4). Thus, whereas early commitment to the exocrine lineage (marked by persistent Ptf1-p48 expression) is permitted, normal acinar cell differentiation was prevented in the setting of an active Notch pathway.

These gain-of-function studies suggest that Notch is capable of inhibiting exocrine differentiation in a cell autonomous manner. Based on the observed expression of Hes1 in Ptf1-p48-positive, amylase-negative E13.5 exocrine progenitors, it is likely that this influence reflects a similar role for endogenous Notch during normal pancreatic development. However, as described above, loss-of-function studies necessary to support this conclusion have been relatively uninformative in the mouse, as excessive commitment to the primary endocrine cell fate and early embryonic lethality preclude evaluation of a later effect of Notch in the exocrine lineage. In order to further examine the effect of Notch on differentiation of exocrine

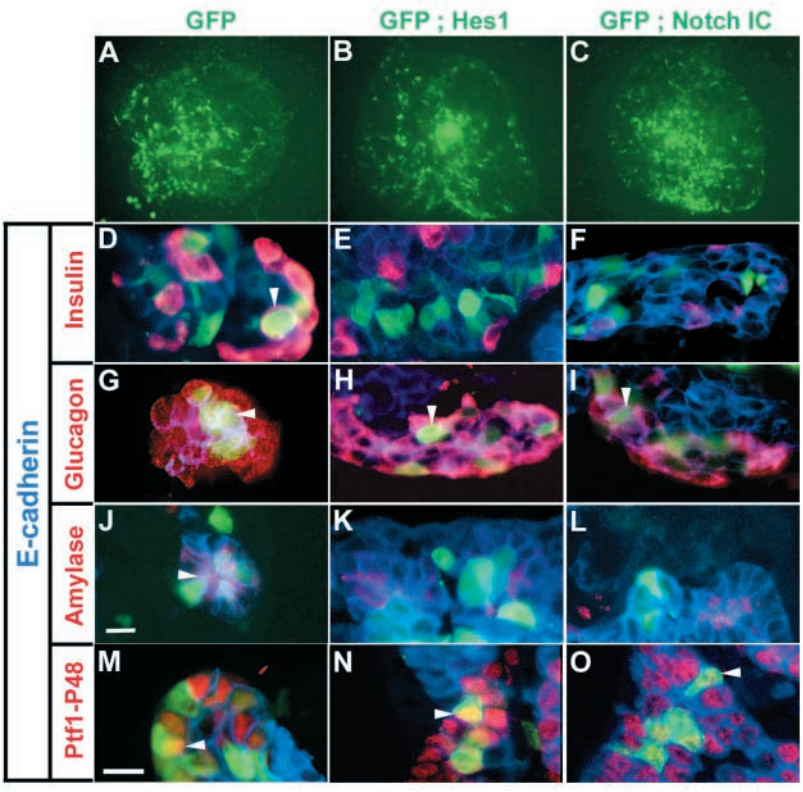
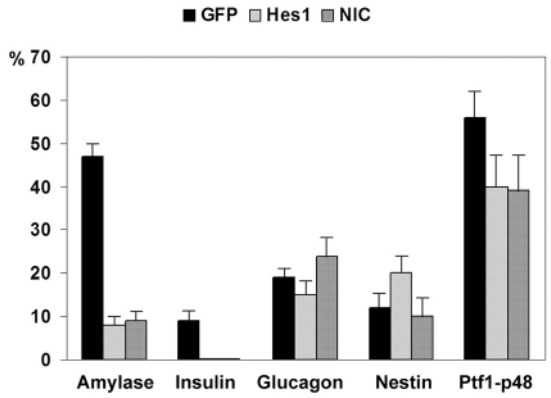


Fig. 2. Effect of ectopic Notch activation on endocrine and exocrine differentiation in explant cultures of E10.5 dorsal pancreatic buds. E10.5 dorsal epithelial buds were infected with lentiviral vectors encoding either GFP alone, GFP and Hes1, or GFP and Notch1-IC, then recombined with mesenchyme and allowed to differentiate in vitro. (A-C) Examination of intact buds demonstrates mosaic GFP expression on day 1 following lentiviral infection. (D-O) Deconvolution fluorescent microscopy demonstrating expression of endocrine and exocrine markers in infected E-cadherin-positive epithelial cells marked by GFP expression, assessed on day 6 following lentiviral infection. Cells infected with GFP alone are able to complete either endocrine or exocrine differentiation programs, as assessed by co-expression of GFP with insulin (D), glucagon (G), amylase (J), or Ptf1-P48 (M). Cells infected with GFP in combination with either Hes1 or Notch1-IC maintain the capacity for alpha-cell differentiation, as evidenced by co-expression of GFP and glucagon (H,I), but are unable to undergo beta-cell differentiation, indicated by absence of cells co-expressing GFP and insulin (E,F). Cells infected with GFP in combination with either Hes1 or Notch1-IC are also unable to undergo acinar cell differentiation, as evidenced by absence of cells co-expressing GFP and amylase (K-L). In contrast, expression of Ptf1-P48 is preserved in cells expressing GFP in combination with either Hes1 or Notch1-IC (N,O). Arrowheads in D,G,H-J,M-O indicate examples of individual cells co-expressing GFP and indicated marker. Scale bars: 10 μm.



	GFP	Hes1	Notch^{IC}
Amylase	**47% ±3**	**8% ±2***	**9% ±2***
	376/802	53/680	44/480
Insulin	**9% ±2**	**<1%***	**0%***
	53/596	3/517	0/347
Glucagon	**19% ±3**	**15% ±3**	**24% ±4**
	112/596	79/517	83/347
Nestin	**12% ±3**	**20% ±4***	**10% ±4**
	41/358	65/330	17/170
Ptf1-p48	**56% ±6**	**40% ±7***	**39% ±8***
	113/206	66/163	52/133

Fig. 3. Quantitative effects of ectopic Notch activation on endocrine and exocrine differentiation in explant cultures of E10.5 dorsal pancreatic buds. The frequency of endocrine and exocrine differentiation was determined by direct counting of GFP-positive epithelial cells in epithelial buds infected with lentiviral vectors encoding either GFP alone, GFP and Hes1, or GFP and Notch1-IC. Top, graphical representation depicting frequency of amylase, insulin, glucagon, nestin and Ptf1-P48 expression in cells infected with indicated vector. Y-axis indicates fraction of GFP-positive, E-cadherin-positive epithelial cells expressing given marker. Bottom, numerical data generated by direct cell counts. Values indicate mean (%)±s.e.m. (bold), as well as corresponding absolute cell counts. Both acinar and beta cell differentiation are prevented by Notch pathway activation, reflected by significant reductions in the frequency of cells expressing either amylase or insulin following infection with either GFP;Hes1 or GFP;Notch1-IC compared with GFP alone. In contrast, the frequency of cells expressing glucagon, nestin or Ptf1-P48 are affected to lesser degrees. Asterisks indicate $P<0.05$ compared with GFP alone, as determined by two-sample Z-test.

pancreas in the absence of confounding effects on early endocrine commitment, we pursued both gain-of-function and loss-of-function studies in developing zebrafish embryos.

Effects of ectopic Notch activation in developing zebrafish pancreas

In contrast to mammalian pancreas, in which endocrine and exocrine cell types are initially recruited from a common progenitor pool, zebrafish pancreas development appears to be characterized by spatially segregated endocrine and exocrine precursor populations (Field et al., 2003; Lin et al., 2004). Zebrafish pancreatic tissue is first apparent as bilateral rows of *pdx1*-positive, *insulin*-positive endocrine cells which coalesce in the midline to form the principal islet by 24 hpf (Argenton et al., 1999; Biemar et al., 2001; Huang et al., 2001). Committed exocrine progenitors are not apparent at this time, but subsequently appear as *ptf1a-p48*-positive cells arising in left lateral endoderm, separated from developing endocrine pancreas (Lin et al., 2004). The relative independence of exocrine and endocrine precursors in developing zebrafish pancreas has been further clarified by examination of gene knockdown phenotypes following injection of antisense morpholinos targeting either *ptf1a-p48* or *pdx1*. *ptf1a-p48* morphants fail to develop differentiated exocrine cells, but undergo entirely normal development of the principal islet. Conversely, *pdx1* morphants display significantly disrupted islet development, even while the initial expression of *ptf1a-p48* remains unaffected (Lin et al., 2004).

To confirm that Notch pathway activation was similarly capable of inhibiting exocrine differentiation in developing zebrafish pancreas, we pursued gain-of-function analysis utilizing transgenic embryos expressing a heat shock-inducible *hsp70:Gal4* transgene, either alone or in combination with a Gal4-responsive *UAS:notch1aICD* allele (Scheer et al., 2001). The *UAS:notch1aICD* transgene was induced by embryo transfer to 40°C media for 30 minutes, and sustained by repeated heat shocks at 10-hour intervals. In order to determine the effect of ectopic Notch pathway activation on differentiation of exocrine pancreas, heat shock was initiated at either 24 hpf (prior to onset of *ptf1a-p48* expression) or at 34 hpf (after onset of *ptf1a-p48* expression, but prior to onset of *trypsin* expression). When heat-shock was initiated at 24 hpf, *hsp70:Gal4* and *hsp70:Gal4;UAS:notch1aICD* embryos remained grossly indistinguishable (Fig. 5), and displayed normal development of liver and foregut as assessed by

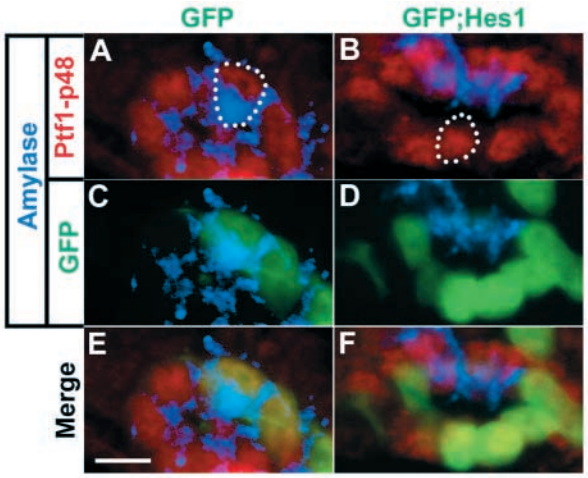


Fig. 4. Ectopic Notch activation expands a population of Ptf1-p48-positive, amylase-negative cells in explant cultures of E10.5 dorsal pancreatic buds. E10.5 dorsal epithelial buds were infected with lentiviral vectors encoding either GFP alone or GFP and Hes1, then recombined with mesenchyme and allowed to differentiate in vitro. (A,C,E) Deconvolution fluorescent microscopy demonstrating patterns of Ptf1-p48 and amylase expression following infection with GFP alone. (B,D,F) Expression patterns following infection with GFP;Hes1. Note apical amylase expression in majority of Ptf1-p48 cells infected with GFP alone, but expansion of a Ptf1a-p48-positive, amylase-negative population in cells infected with GFP;Hes1. Broken lines in A and B indicate contour of representative GFP-positive, Ptf1-p48-positive cells either expressing (A) or lacking amylase (B). A similar pattern is observed following infection with GFP;Notch1-IC. Scale bar: 10 μm.

examination of *hhex* and *GATA6* expression (data not shown). In contrast, subtle but highly reproducible effects on both exocrine and endocrine differentiation were observed in the setting of forced Notch pathway activation. As assessed at 32 hpf, shortly after the normal onset of *ptf1a-p48* expression, 4% (1/23) of *hsp70:Gal4;UAS:notch1aICD* embryos demonstrated endodermal expression of *ptf1a-p48*, compared with 68% (25/37) in *hsp70:Gal4* controls (Fig. 5A,B; $P<0.001$). This delay in *ptf1a-p48* expression was short-lived; by 34 hpf, endodermal expression of *ptf1a-p48* was uniformly present in both groups, and remained normal at later time points (Fig. 5C,D). In spite of this rapid recovery in *ptf1a-p48* expression, the onset of *trypsin* expression was significantly delayed in *hsp70:Gal4;UAS:notch1aICD* embryos. As assessed at 44 hpf, only 42% (21/50) of *hsp70:Gal4;UAS:notch1aICD* embryos showed detectable *trypsin* transcripts at 44 hpf, compared with 89% (39/44) of *hsp70:Gal4* sibling controls (Fig. 5E,F; $P<0.001$).

With respect to effects on endocrine differentiation, 12% (2/17) of *hsp70:Gal4;UAS:notch1aICD* embryos heat-shocked at 24 hpf demonstrated a normal pattern of *insulin* expression at 32 hpf, compared with 88% (14/16) of *hsp70:Gal4* controls ($P<0.001$). By 44 hpf, 0/21 *hsp70:Gal4;UAS:notch1aICD* embryos had normal *insulin* expression, compared with 21/21 *hsp70:Gal4* controls (Fig. 5G,H). This loss of *insulin* expression occurred in the setting of preserved expression of *pdx1*; no differences in the location, intensity or distribution of *pdx1* transcripts were observed between *hsp70:Gal4* and *hsp70:Gal4;UAS:notch1aICD* embryos at either 32 or 44 hpf (Fig. 5I,J). These data suggest that ectopic Notch activation is capable of inhibiting both the onset of acinar cell differentiation and the maintenance of beta cell differentiation in developing zebrafish embryos.

Based on the ability of Notch to block acinar cell differentiation in developing mouse pancreas even in the face of preserved *ptf1a-p48* expression, we next examined the effects of delayed Notch pathway activation initiated after the appearance of *ptf1a-p48*, but prior to the onset of *trypsin* expression. For these studies, heat shock was applied at 34 hpf, at which point all embryos express *ptf1a-p48* in developing endoderm. Following heat shock at 34 hpf and harvest at 44 hpf, both *hsp70:Gal4* and *hsp70:Gal4;UAS:notch1aICD* embryos demonstrated normal patterns of *hhex*, *GATA6*, *pdx1* and *insulin* expression (data not shown). The observed maintenance of insulin expression following induction of *notch1aICD* at 34 hpf, but not 24 hpf, suggests differential sensitivity between early and mature endocrine cells with respect to ectopic Notch activation, similar to that reported in the mouse (Murtaugh et al., 2003). As expected, a normal pattern of *ptf1a-p48* expression was also evident in both groups following heat shock at 34 hpf (Fig. 5M,N). However, delayed Notch activation again resulted in a significant delay in acinar cell differentiation, with trypsin transcripts present in 88% (64/73) of *hsp70:Gal4* controls, but only 53% (32/60) of *hsp70:Gal4;UAS:notch1aICD* embryos (Fig. 5K,L; $P<0.001$).

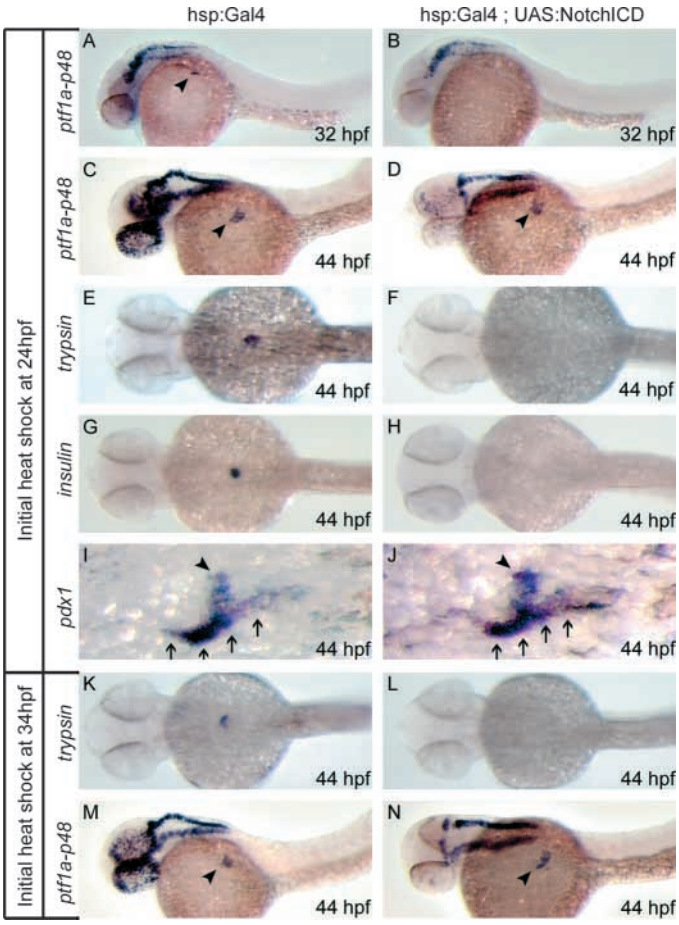


Fig. 5. Notch pathway activation delays exocrine differentiation in developing zebrafish pancreas. Heat-shocked embryos expressing a *hsp:Gal4* transgene alone or in combination with *UAS:notch1aICD* were assessed for initiation of *ptf1a-p48* and *trypsin* expression, as well as expression of *insulin* and *pdx1*. Following heat shock at 24 hpf, *ptf1a-p48* expression is delayed in 32 hpf *hsp:Gal4;UAS:notch1aICD* embryos (B) compared with *hsp:Gal4* controls (A), but recovers by 34 hpf (data not shown). As assessed at 44 hpf, both *trypsin* (E,F) and *insulin* (G,H) expression are reduced in *hsp:Gal4;UAS:notch1aICD* embryos compared with *hsp:Gal4* controls, even while *ptf1a-p48* (C,D) and *pdx1* (I,J) expression remain normal. Following delayed heat shock initiated at 34 hpf (after normal onset of *ptf1a-p48* expression), *ptf1a-p48* expression remains normal at all time points (M,N), whereas expression of *trypsin* is delayed (K,L). Arrowheads in A,C,D,M,N indicate endodermal domain of *ptf1a-p48* expression, distinct from expression in developing hindbrain. Arrowheads in I and J indicate pdx1-positive principal islet; arrows indicate adjacent pdx1-positive intestine.

Loss of Notch signaling accelerates exocrine differentiation in developing zebrafish pancreas

In order to determine if the inhibitory effect of Notch on exocrine differentiation reflected an influence normally exerted by endogenous Notch pathway components, we utilized two different methodologies to generate Notch loss-of-function phenotypes. Fish bearing homozygous nonsense mutations at the *mindbomb* locus (*mib*^{ta52b}) lack a ubiquitin ligase required for normal post-translational processing and trafficking of delta, resulting in defective Notch pathway activation (Itoh et al., 2003). A similar phenotype can be generated by injection of single cell embryos with RNA encoding a fusion protein comprised of GFP in frame with a dominant-negative *Suppressor of Hairless* DNA binding mutant (GFPdnSuH) (Wettstein et al., 1997). To determine the impact of defective

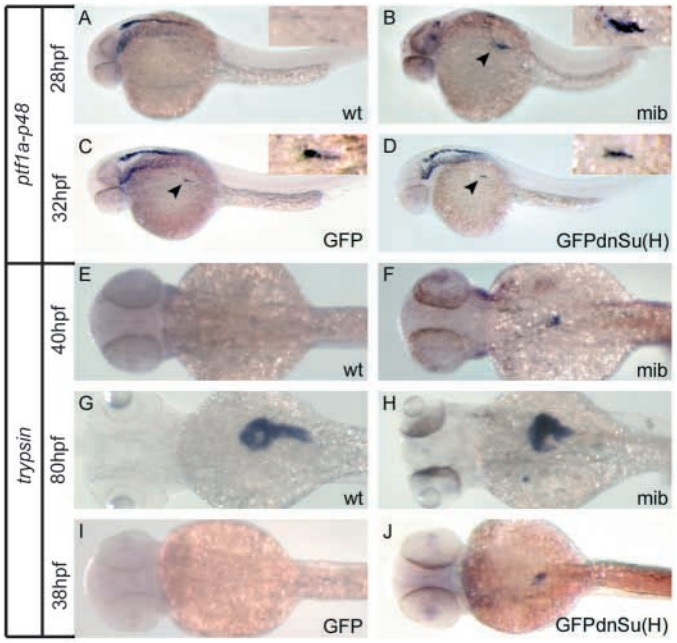


Fig. 6. Defects in Notch pathway activation result in acceleration of exocrine differentiation in developing zebrafish pancreas. (A-D) *Mindbomb* (mib) mutant embryos (B) and embryos injected with RNA encoding a dominant-negative *Suppressor of Hairless* DNA binding mutant [GFPdnSu(H)] (D) show either accelerated (mib) or normal [GFPdnSu(H)] onset of *ptf1a-p48* expression compared with clutchmate controls (A,C). Arrows indicate endodermal domain of *ptf1a-p48* expression, distinct from expression in developing hindbrain. Insets in A-D show magnified view of endodermal *ptf1a-p48* expression domain. (E-J) *mindbomb* (mib) mutant embryos (F,H) and embryos expressing GFPdnSu(H) (J) show accelerated acinar cell differentiation compared with clutchmate controls (E,G,I), marked by early onset of trypsin expression. Note normal absence of trypsin expression in control embryos at 40 hpf (E,I), but accelerated onset of trypsin expression in mib$^{ta52/ta52}$ and GFPdnSu(H)-injected embryos (F,J). At 80 hpf, the size and contour of established trypsin-positive exocrine parenchyma is also altered in mib$^{ta52/ta52}$ embryos compared with wild-type clutchmates (G,H). Wt indicates wild-type clutchmates arising from mib$^{ta52/wt}$ x mib$^{ta52/wt}$ cross. GFP indicates clutchmate control embryos injected with RNA encoding GFP alone.

Notch pathway activation on differentiation of exocrine pancreas, we compared the timing of exocrine differentiation in *mib*$^{ta52b/ta52b}$ embryos, wild-type clutchmates, GFPdnSuH-injected embryos, and control embryos injected with RNA encoding GFP alone. In both *mib*$^{ta52b/ta52b}$ and GFPdnSuH-injected embryos, loss of Notch function was confirmed by generation of a neurogenic phenotype revealed by both HuC staining (data not shown) and by gross morphology. Embryos were collected across an extended time course designed to span the period before and after the normal onset of endodermal *ptf1a-p48* expression (24-40 hpf), as well as the period before and after the normal onset of *trypsin* expression (36-52 hpf).

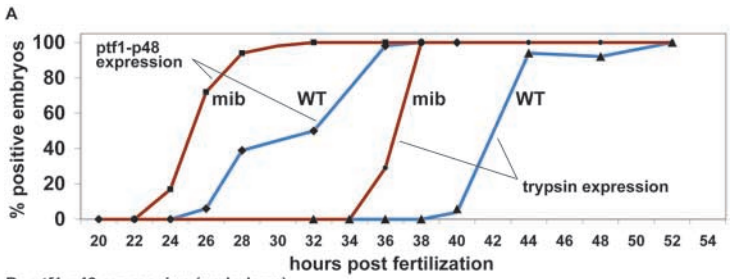
For both *ptf1a-p48* and *trypsin*, the onset of expression was accelerated in *mib*$^{ta52b/ta52b}$ embryos compared with phenotypically wild-type (either *mib*$^{ta52b/wt}$ or *mib*$^{wt/wt}$) clutchmates, consistent with a normal inhibitory role for endogenous Notch in regulating exocrine differentiation (Fig. 6A,B,E-H, Fig. 7). The effect was especially pronounced for *trypsin*, with 100% of *mib*$^{ta52b/ta52b}$ embryos showing expression at 40 hpf, whereas only 4% of wild-type fish had detectable *trypsin* transcripts at this time point (Fig. 6; $P<0.001$). In contrast, no acceleration of endocrine differentiation was apparent in *mib*$^{ta52b/ta52b}$ embryos, as assessed by evaluating the onset of *pdx1* and *insulin* expression (data not shown).

A similar accelerated onset of *trypsin* expression (but not *ptf1a-p48*) was observed in GFPdnSuH-injected embryos compared with GFP-injected controls (Fig. 6C,D,I,J). At 30 hpf, 33/56 GFPdnSuH-injected embryos (59%) and 23/47 control embryos (49%) had *ptf1a-p48* transcripts detected in developing endoderm ($P=0.31$). By 38 hpf, *trypsin* expression had been initiated in 35/71 GFPdnSuH-injected embryos (49%), but only 4/58 controls (7%; $P<0.001$). These results suggest an exocrine 'pancreatogenic' phenotype in the absence of effective Notch signaling, and define inhibition of exocrine pancreatic differentiation as a normal function of endogenous Notch pathway activation.

Notch inhibits activity of the Ptf1 transcriptional complex

In many systems, Notch-mediated inhibition of cellular differentiation involves downregulated expression of lineage-specifying transcription factors (Beatus and Lendahl, 1998; Fisher and Caudy, 1998; Kuroda et al., 1999; Lee et al., 2001).

A



B ptf1-p48 expression (endoderm)

	24 hpf	26 hpf	28 hpf	32 hpf	36 hpf	40 hpf
WT	0/25	2/34 (6%)	12/31 (39%)	18/35 (51%)	53/56 (95%)	12/12 (100%)
mib	2/12 (17%)	13/18 (72%)	17/18 (94%)	23/23 (100%)	18/18 (100%)	5/5 (100%)

C trypsin expression

	36 hpf	38 hpf	40 hpf	44 hpf	48 hpf	52 hpf
WT	0/46	0/16	3/76 (4%)	32/34 (94%)	44/48 (92%)	59/59 (100%)
mib	5/17 (29%)	3/3 (100%)	20/20 (100%)	15/15 (100%)	17/17 (100%)	25/25 (100%)

Fig. 7. Frequency of *ptf1a-p48* and *trypsin* expression in developing wild-type and *mib*$^{ta52/ta52}$ zebrafish embryos. (A) Graph depicting fraction of embryos expressing either *ptf1a-p48* or *trypsin* at indicated developmental time points, as determined by whole-mount in situ hybridization. (B,C) Raw numerical data generated by analysis of more than 600 embryos. Mib indicates *mib*$^{ta52/ta52}$ embryos; WT indicates wild-type clutchmate controls.

Based on these precedents, we initially predicted that Notch pathway activation would be associated with downregulated expression of *Ptf1-P48*. In both mouse and zebrafish model systems, however, *Ptf1-P48* expression was preserved in the setting of forced Notch pathway activation, even while acinar cell differentiation was prevented or delayed. Based on the additional observation that Notch activation was capable of delaying exocrine differentiation in zebrafish pancreas even when initiated after the normal onset of *Ptf1-P48* expression, we considered that Notch might inhibit the functional activity of the Ptf1 transcriptional complex, comprised of the Class II HLH Ptf1-p48 protein and a Class I E-box binding partner (Obata et al., 2001; Rose et al., 2001). As an initial assessment of the effect of Notch on Ptf1 activity, we performed electrophoretic mobility shift assays with nuclear extracts from the AR42J rat acinar cell carcinoma line, using a labeled oligonucleotide corresponding to the A element of the rat *elastase 1* enhancer (Rose et al., 2001). In this context, a positive gel shift results from endogenous Ptf1 DNA-binding activity, with specificity further confirmed by supershift following addition of anti-Ptf1-p48 antibody (Fig. 8A). Endogenous Ptf1 DNA-binding activity was inhibited following forced Notch pathway activation, accomplished using bicistronic adenoviral vectors encoding either GFP alone or in combination with Notch1-IC. In addition, activation of selective Notch pathway components was accomplished using adenoviral vectors encoding GFP in combination with individual Notch/Su(H) target genes, including Hes1, Hey1 and Hey2. In these experiments, activated Notch1-IC, Hes1 and Hey1 significantly reduced Ptf1 DNA-binding activity, whereas Hey2 had no effect (Fig. 8A). Northern blot analysis demonstrated associated downregulation of *elastase-1* expression, but inconsistent changes in expression of *Ptf1-P48* (data not shown), suggesting that Notch pathway activation may alter Ptf1 function, independent of associated changes in Ptf1-p48 expression.

In order to study the influence of Notch and Notch target genes on Ptf1 function, independent of changes in the expression levels of Ptf1 component proteins, we conducted co-transfection experiments in COS7 cells using a Ptf1-responsive luciferase reporter containing a 4X tandem repeat of the Ptf1 binding element from the rat chymotrypsinogen promoter (Ptf1-luc). Cells were transfected with pcDNA-based expression vectors encoding Ptf1-p48 and E47, allowing us to

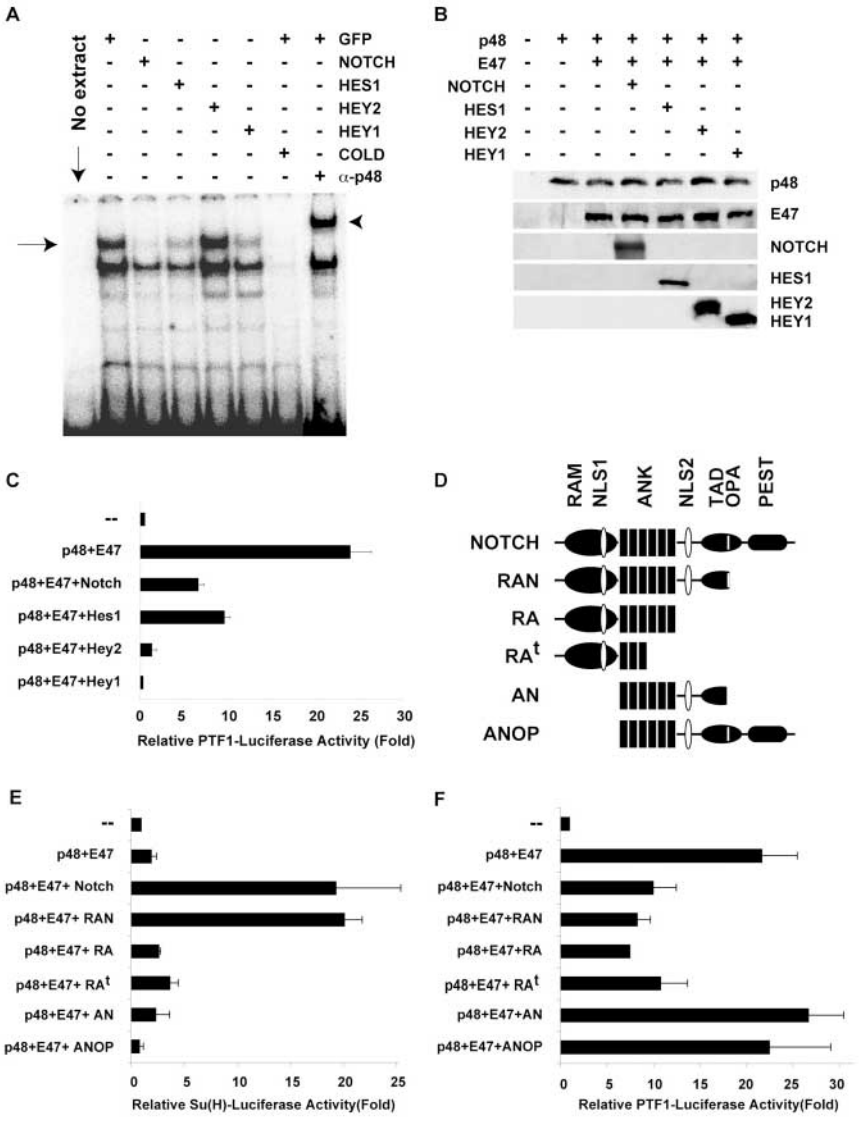


Fig. 8. Notch pathway activation inhibits activity of the Ptf1 transcriptional complex.
(A) Electrophoretic mobility shift assay measuring endogenous Ptf1 DNA-binding activity in nuclear extracts of rat AR42J cells infected with adenoviral vectors encoding GFP alone or in combination with either Notch1-IC, Hes1, Hey2 or Hey1 as indicated. Upper band (arrow) represents specific binding of oligonucleotide corresponding to the A element of rat elastase promoter, confirmed by supershift in the presence of anti-Ptf1-p48 antiserum (arrowhead). Cold indicates presence of excess unlabelled oligonucleotide. Note inhibition of endogenous Ptf1 DNA-binding activity by Notch1-IC, Hes1 and Hey1. (B) Western blot analysis of COS7 extracts following co-transfection of Ptf1-P48 alone or in combination with E47 and either Notch1-IC, Hes1, Hey2 or Hey1. Note constant level of Ptf1-p48 and E47 protein across conditions, allowing analysis of Ptf1 activity (C) in the absence of associated changes in component protein levels.
(C) Corresponding determination of Ptf1 activity as assessed by activation of Ptf1-responsive luciferase reporter (Ptf1-luc). Notch1-IC, Hes1, Hey2 and Hey1 effectively inhibit Ptf1 activity, independent of associated changes in protein levels of Ptf1-p48 or E47. (D) Schematic depiction of Notch1-IC truncation mutants used in E and F, below. NLS indicates nuclear localization signal. ANK indicates ankyrin repeats. (E) Ability of Notch1-IC and Notch1-IC truncation mutants to activate Su(H)-responsive luciferase reporter [Su(H)-luc].
(F) Corresponding ability of Notch1-IC and Notch1-IC truncation mutants to inhibit Ptf1-p48/E47-mediated activation of Ptf1-responsive luciferase reporter (Ptf1-luc). Ability to inhibit Ptf1 resides within N-terminal domains. (–) in C,E,F indicates transfection of reporter construct alone.

effectively clamp Ptf1 component protein levels at a constant level while we altered expression of Notch1-IC and Notch/Su(H) target genes (Fig. 8B). As expected, COS7 cells exhibited little endogenous Ptf1 activity, reflecting their non-pancreatic identity (Fig. 8C). Co-transfection of Ptf1-p48 and E47 resulted in a 20- to 25-fold increase in Ptf1-luc activity, reflecting effective expression of exogenous Ptf1 components. The addition of activated Notch1-IC resulted in >50% reduction in normalized Ptf1-luc activity, without associated changes in Ptf1-p48 or E47 protein levels. Similar reductions in Ptf1 activity were observed following co-transfection with either Hes1, Hey1 or Hey2. The ability of Hey2 to reduce Ptf1 transcriptional activation but not DNA binding activity suggests that Notch target genes may inhibit Ptf1 function through multiple mechanisms, some of which may not involve displacement of the Ptf1 complex from cognate DNA sequences. In order to determine whether the ability of Notch1-IC to downregulate Ptf1 function was tightly linked to Su(H)-dependent transcriptional activation, a series of Notch1-IC deletion mutants lacking either the N-terminal RAM domain or C-terminal OPA and PEST domains was employed (Fig. 8D). These studies revealed that the ability of Notch1-IC to inhibit Ptf1 function resided within N-terminal elements, and did not necessarily correlate with the ability to maximally activate a Su(H)-responsive luciferase reporter (Fig. 8E,F). Together, these data demonstrate that activated Notch is able to downregulate Ptf1 function despite ongoing expression of Ptf1 component proteins, representing a probable mechanism for the observed in vivo effects of Notch.

Discussion

During mammalian pancreatic development, committed endocrine and exocrine progenitors are sequentially recruited from a common precursor population in developing pancreatic epithelium. Reflecting this common origin, endocrine and exocrine differentiation typically represent competing cell fates within a limited precursor pool, with changes in early endocrine differentiation typically reflected by inverse changes in the later appearing exocrine cell mass. Although the role of Notch signaling in regulating early endocrine differentiation has been well established (Apelqvist et al., 1999; Jensen et al., 2000b), the role of endogenous Notch pathway activation in the regulation of subsequent exocrine differentiation has been more difficult to determine. Several recent studies have suggested that Notch is indeed capable of altering the ability of pancreatic epithelial cells to achieve and maintain an acinar cell identity. When a Notch1-IC transgene is activated in developing mouse pancreas using *pdx1* promoter elements, both endocrine and exocrine differentiation are repressed (Hald et al., 2003; Murtaugh et al., 2003), consistent with an inhibitory role for Notch in regulating differentiation in both lineages. When Notch1-IC is misexpressed in adult exocrine pancreatic tissue, mature acinar cells are replaced by a nestin-positive precursor population, through a process of apparent dedifferentiation (Miyamoto et al., 2003). Combined with the current results, these studies suggest that endogenous Notch pathway activation acts to regulate the sequential recruitment of endocrine and exocrine cell types from a common precursor pool in developing mouse pancreas. In this regard, lineage specification during mammalian pancreas development may be viewed as sharing features in common with *Drosophila* sensory bristle development (Posakony, 1994) and mammalian hematopoiesis (Anderson et al., 2001; Ohishi et al., 2003), in which sequential cell fate decisions are regulated by Notch activity.

Although the competitive and sequential recruitment of endocrine and exocrine cell types from a common precursor pool in developing mouse pancreas has made it difficult to discern the inhibitory influence of endogenous Notch pathway components on exocrine differentiation, this influence is apparent in developing zebrafish pancreas, where endocrine and exocrine precursors appear to arise independently (Field et al., 2003; Lin et al., 2004). Thus, while accelerated exocrine differentiation is not observed following targeted inactivation of Notch pathway components in the mouse (Apelqvist et al., 1999; Jensen et al., 2000b), this phenomenon is observed in developing zebrafish pancreas in the setting of either inactivating *mindbomb* mutations or expression of dominant-negative *Su(H)*. These findings clearly define a role for endogenous Notch pathway components in regulating zebrafish exocrine pancreatic differentiation. Based on observed co-expression of Hes1 and Ptf1-p48 in E13.5 mouse pancreas, the absence of Hes1 expression in differentiated acinar cells, the ability of either Hes1 or Notch1-IC to inhibit exocrine differentiation in a cell autonomous manner, and the ability of either Notch1-IC or Notch/Su(H) target genes to inhibit Ptf1 activity, it is likely that this influence is also active in the mouse.

In both mouse and zebrafish, we frequently observed ongoing expression of *Ptf1-P48* in the setting of either endogenous or ectopic Notch activation, even while acinar cell differentiation was prevented. In addition, we observed that activated Notch and Notch/Su(H) target genes were able to downregulate Ptf1 activity even in the face of ongoing Ptf1-p48 protein expression. Thus the respective mechanisms by which Notch inhibits endocrine and exocrine differentiation appear to be fundamentally different, with inhibition of endocrine differentiation achieved at least in part by blocking *Ngn3* expression (Hald et al., 2003; Jensen et al., 2000a; Murtaugh et al., 2003), whereas inhibition of exocrine differentiation is achieved by blocking Ptf1 function in the face of ongoing *Ptf1-P48* expression. This difference probably reflects the known requirement for *Ptf1-P48* in early pancreatic development, prior to its role in promoting acinar cell differentiation. In developing mouse pancreas, *Ptf1-P48* expression is detectable as early as E9.5 (Chiang and Melton, 2003; Kawaguchi et al., 2002; Obata et al., 2001), well before the presumed onset of Ptf1-dependent zymogen expression. In addition, rigorous lineage tracing studies and further characterization of the *Ptf1-P48*-null phenotype have demonstrated that *Ptf1-P48*-positive precursor cells broadly contribute to both exocrine and endocrine lineages in developing mouse pancreas, and that *Ptf1-P48* is required for critical events in early pancreatic development (Kawaguchi et al., 2002). It is intriguing to consider that these early functions of *ptf1a-p48* may be mediated through mechanisms not involving activity of the Ptf1 transcriptional complex, and that Notch may function not only to reserve an undifferentiated precursor pool, but also to inhibit Ptf1-dependent aspects of *Ptf1-P48* activity until that point when *Ptf1-P48* expression becomes restricted to dedicated acinar cells. Thus, while the initial influence of *Ptf1-P48* on early pancreatic morphogenesis appears to occur in the setting of an active Notch pathway and low levels of Ptf1 transcriptional activity, subsequent Ptf1-

dependent functions require silencing of Notch activity within the exocrine lineage. Based on this interpretation, the identification of mechanisms underlying Notch-resistant, Ptf1-independent aspects of *Ptf1-P48* activity represents an important area for future research.

In attempting to synthesize the current results with prior studies of lineage relationships and Notch signaling in developing mouse pancreas (Apelqvist et al., 1999; Chiang and Melton, 2003; Gu et al., 2003; Gu et al., 2002; Hald et al., 2003; Jensen et al., 2000a; Jensen et al., 2000b; Kawaguchi et al., 2002; Murtaugh et al., 2003), we have generated the working model depicted in Fig. 9. This model emphasizes both the sequential and competitive recruitment of endocrine and exocrine cell types from a common progenitor pool, as well as the iterative influence of Notch signaling in regulating these events. Under this model, silencing of Notch signaling within a common *Ptf1-P48*-positive precursor pool is initially associated with endocrine lineage commitment, marked by the onset of *Ngn3* expression. In *Ptf1-P48*-positive cells with ongoing Notch activity, the effects of *Ptf1-P48* on pancreatic morphogenesis continue to be realized, even while Ptf1-dependent acinar cell differentiation is prevented. Upon subsequent silencing of Notch on E14.5, widespread acinar cell differentiation ensues. Although the model assumes the predominantly sequential recruitment of endocrine and exocrine cell types from a common progenitor pool, it must be noted that these processes are characterized by considerable temporal overlap, and that definitive markers indicating 'irreversible' commitment to either the endocrine or exocrine lineages have not yet been identified.

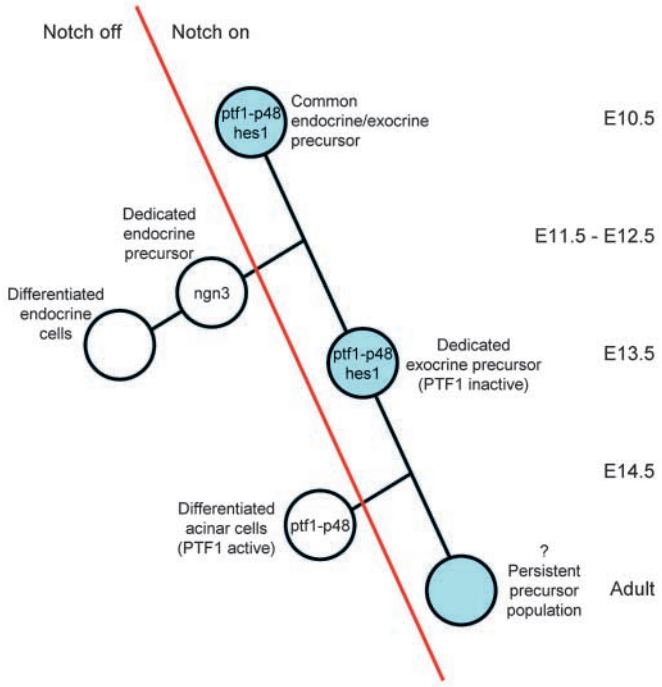


Fig. 9. Notch regulates sequential recruitment of endocrine and exocrine cell types in developing mouse pancreas. Simplified model summarizes current results within the context of known lineage relationships (Chiang and Melton, 2003; Gu et al., 2003; Gu et al., 2002; Kawaguchi et al., 2002), as well as prior studies of Notch signaling in developing mouse pancreas (Apelqvist et al., 1999; Hald et al., 2003; Jensen et al., 2000a; Jensen et al., 2000b; Murtaugh et al., 2003). Diagonal red line distinguishes cell types characterized by active Notch signaling, indicated by *Hes1*-positivity, from those with an inactive Notch pathway. Notch pathway activation inhibits early recruitment of *Ngn3*-positive endocrine precursors from a common endocrine/exocrine precursor pool, and also inhibits generation of differentiated acinar cells from dedicated exocrine precursors. Between E10.5 and E12.5, *Ngn3*-positive endocrine precursors are recruited from a *Ptf1-P48/Hes1*-positive common precursor pool. Recruitment of dedicated endocrine precursors in inhibited by Notch, thereby reserving an undifferentiated cell population responsible for ongoing epithelial growth as well as subsequent exocrine differentiation. By E13.5, ongoing *Ptf1-P48* expression marks a dedicated exocrine precursor pool. Ongoing Notch activity within this pool results in an inactive Ptf1 transcriptional complex, allowing terminal acinar cell differentiation to be avoided until Ptf1-independent influences of *Ptf1-P48* on epithelial morphogenesis are fully realized. Notch silencing at E14.5 allows onset of Ptf1 activity and acinar cell differentiation. The persistence of Notch-regulated precursor cells in adult pancreas remains uncertain.

In summary, we find that Notch pathway activation represents a characteristic feature of *Ptf1-P48*-positive exocrine precursors in developing mouse pancreas, and that ectopic Notch activation prevents acinar cell differentiation in a cell autonomous manner. Gain-of-function studies in both mouse and zebrafish suggest that Notch activity prevents exocrine differentiation even in the face of ongoing *Ptf1-P48* expression. Loss of Notch function in developing zebrafish pancreas is associated with accelerated development of exocrine pancreas, defining a normal role for endogenous Notch in the regulation of acinar cell differentiation. Finally, we find that Notch suppresses activity of the Ptf1 complex, independent of changes in the levels of Ptf1 component proteins, representing a probable mechanism for the influence of Notch in developing exocrine pancreas.

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