Lineage tracing reveals the dynamic contribution of $Hes1^+$ cells to the developing and adult pancreas

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SUMMARY
Notch signaling regulates numerous developmental processes, often acting either to promote one cell fate over another or else to inhibit differentiation altogether. In the embryonic pancreas, Notch and its target gene $Hes1$ are thought to inhibit endocrine and exocrine specification. Although differentiated cells appear to downregulate $Hes1$, it is unknown whether $Hes1$ expression marks multipotent progenitors, or else lineage-restricted precursors. Moreover, although rare cells of the adult pancreas express $Hes1$, it is unknown whether these represent a specialized progenitor-like population. To address these issues, we developed a mouse $Hes1^{CreERT2}$ knock-in allele to inducibly mark $Hes1^+$ cells and their descendants. We find that $Hes1$ expression in the early embryonic pancreas identifies multipotent, Notch-responsive progenitors, differentiation of which is blocked by activated Notch. In later embryogenesis, $Hes1$ marks exocrine-restricted progenitors, in which activated Notch promotes ductal differentiation. In the adult pancreas, $Hes1$ expression persists in rare differentiated cells, particularly terminal duct or centroacinar cells. Although we find that $Hes1^+$ cells in the resting or injured pancreas do not behave as adult stem cells for insulin-producing beta ($\beta$)-cells, $Hes1$ expression does identify stem cells throughout the small and large intestine. Together, these studies clarify the roles of Notch and $Hes1$ in the developing and adult pancreas, and open new avenues to study Notch signaling in this and other tissues.

KEY WORDS: Pancreas, $Hes1$, Notch, Stem cell, Mouse

INTRODUCTION
The vertebrate pancreas comprises three major cell types: endocrine islets, which include insulin-producing $\beta$-cells; and a network of exocrine acinar and duct cells, which are responsible for producing and transporting digestive enzymes, respectively. The Notch signaling pathway has been implicated in several aspects of pancreatic cell fate determination, beginning with the finding that mouse embryos lacking various Notch components, including the downstream target gene $Hes1$, exhibit overproduction of endocrine cells (Apelqvist et al., 1999; Jensen et al., 2000). $Hes1$ can repress the promoter of $Neurog3$, a crucial pro-endocrine transcription factor, and de-repression of $Neurog3$ in the absence of $Hes1$ may drive excessive endocrine differentiation (Apelqvist et al., 1999; Jensen et al., 2000; Lee et al., 2001). In gain-of-function experiments, Notch also inhibits exocrine acinar cell development, promoting instead progenitor maintenance (Esni et al., 2004; Hald et al., 2003; Murtaugh et al., 2003). These findings are corroborated by studies in zebrafish (Esni et al., 2004; Yee et al., 2005; Zecchin et al., 2006), and conform to a generic conception of Notch as regulating cell fate throughout animal development (Lai, 2004).

The Notch pathway knockout phenotypes implied that the early pancreas comprised multipotent cells, the differentiation of which was held in check by Notch signaling (Apelqvist et al., 1999; Jensen et al., 2000). Lineage-tracing studies suggest that multipotent progenitors reside in the ‘tips’ of the embryonic pancreatic epithelium, the expansion of which leaves behind ‘trunks’ that give rise to ducts and islets (Kopinke and Murtaugh, 2010; Solar et al., 2009; Zhou et al., 2007). How Notch regulates this process is unknown, although it may signal through $Hes1$ to repress $Neurog3$ (Lee et al., 2001) and control the balance of duct and islet differentiation. Contradicting this model, however, deletion of $Notch1$ and $Notch2$, the major receptors expressed in the pancreas, has little effect on late embryonic islet development (Nakahi et al., 2008).

Whether progenitor cells persist in the adult pancreas, particularly for insulin-producing $\beta$-cells, remains controversial. Two lineage tracing approaches have been taken to address this issue: ‘pulse-chase’ labeling of mature islet cells, to detect changes in labeling frequency caused by differentiation of new $\beta$-cells (neogenesis); or marking acini and/or ducts, to determine whether they can contribute to $\beta$-cells. The former studies argue against $\beta$-cell neogenesis (Dor et al., 2004), and the latter indicate that neogenesis is either non-existent (Desai et al., 2007; Kopinke and Murtaugh, 2010; Solar et al., 2009) or rare (Inada et al., 2008) in the uninjured pancreas. Pancreatic injury, in particular caused by ligation of the main duct, has been proposed to induce facultative neogenesis from acinar or duct cells (Wang et al., 1995; Xu et al., 2008), for which exist both contradictory and supporting lineage tracing data (Inada et al., 2008; Solar et al., 2009). As previous approaches used mature duct or acinar marker genes to drive Cre expression, however, they may have excluded specialized adult progenitor cells. These might include centroacinar cells, terminal elements of the exocrine ductal network in which Notch-$Hes1$ signaling appears particularly high.
(Miyamoto et al., 2003; Parsons et al., 2009; Stanger et al., 2005). These cells have been suggested to generate new β-cells following injury (Hayashi et al., 2003; Nagasao et al., 2003), and they can give rise to both acinar and islet cells following isolation and culture (Rovira et al., 2010).

To understand how and when Notch-Hes1 signaling regulates pancreatic progenitor cells, we generated ‘knock-in’ mice in which the tamoxifen-dependent CreERT2 recombinase is targeted to the Hes1 locus. With these mice, we have analyzed the stage-specific differentiation potential of Notch-responsive cells in the embryonic pancreas, revealing a novel shift from multipotent to exocrine-restricted progenitor cells. This parallels a shift in the cellular response to Notch, from arresting differentiation to promoting duct cell specification. In the adult, we found that Hes1+ duct and centroacinar cells appear to be fixed in their fate, and do not detectably contribute to β-cells, even after duct ligation injury. Ours is the first study to address the fate of Notch-responsive cells in any adult tissue, and supports an emerging model that lineage boundaries in the pancreas are normally fixed at birth.

MATERIALS AND METHODS

Mice

We used bacterial recombineering (Liu et al., 2003) to generate a Hes1 (CreERT2-neo) targeting vector, in which most of the Hes1 open reading frame, including the 5’HLH domain, is replaced by that of CreERT2 (Feil et al., 1997), linked to an FRT-flanked neo cassette (see Fig. S1A in the supplementary material). This was electroporated into R1 ES cells (Nagy et al., 1993), generously provided by Mario Capecchi (University of Utah, USA), and G418-resistant ES cell clones were screened by Southern blotting and PCR (see Fig. S1B in the supplementary material and data not shown). Germline chimeras were derived by the University of Utah Transgenic Core Facility. The neo cassette was excised in vivo by breeding to Rosa26RFP (Farley et al., 2000), obtained from the Jackson Laboratory. Cre reporter mice R26R<sup>ERT2</sup> (Srinivas et al., 2001) and R26R<sup>2a</sup> (Soriano, 1999) were obtained from the Jackson Laboratory. Rosa26<sup>WTC</sup> (Murtaugh et al., 2003) and Pdx1Cre mice (Gu et al., 2002) were provided by Doug Melton (Harvard University, MA, USA). Ctnnb1<sup>lox(ex3)</sup> mice (Harada et al., 1999) were provided by Makoto Mark Taketo (Kyoto University, Japan). Tamoxifen (Sigma T-5648) was purchased from the Transgenic Core Facility. The targeting vector, in which most of the Hes1 locus. With these mice, we have analyzed the stage-specific differentiation potential of Notch-responsive cells in the embryonic pancreas, revealing a novel shift from multipotent to exocrine-restricted progenitor cells. This parallels a shift in the cellular response to Notch, from arresting differentiation to promoting duct cell specification. In the adult, we found that Hes1+ duct and centroacinar cells appear to be fixed in their fate, and do not detectably contribute to β-cells, even after duct ligation injury. Ours is the first study to address the fate of Notch-responsive cells in any adult tissue, and supports an emerging model that lineage boundaries in the pancreas are normally fixed at birth.

Immunostaining and lineage analysis

Tissues were fixed and immunostained essentially as described previously (Kopinke and Murtaugh, 2010). Immunofluorescence was performed on frozen sections (7-8 µm) of tissue fixed in 4% paraformaldehyde/PBS (4°C, 1-2 hours). Other analyses used paraffin sections (6 µm) of tissue fixed in zinc-buffered formalin (room temperature, overnight). Primary antibodies used in this study are listed in Table 1; where indicated, sections were also stained with Dolichos biflorus agglutinin (DBA) lectin (Vector Laboratories), which marks duct cells (Kobayashi et al., 2002). Staining was analyzed by compound fluorescent or light microscopy, using MicroSuite software (Olympus). Photomicrographic images were processed using Adobe Photoshop, with parallel images processed identically.

Table 1. Primary antibodies used in this study

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RESULTS

Hes1 expression and gene targeting

To extend previous studies of Hes1 expression (Apeîqvist et al., 1999; Esni et al., 2004; Jensen et al., 2000; Lammert et al., 2000), we performed Hes1 immunostaining on pancreata of different embryonic stages, spanning major developmental transitions. At E11.5, when most cells are undifferentiated progenitors, we found widespread but non-uniform expression of Hes1 in the epithelium (Fig. 1A), consistent with previous immunostaining and in situ hybridization studies (Jensen et al., 2000; Lammert et al., 2000; Murtaugh et al., 2005; Nakhai et al., 2008). We observed Hes1 downregulation from E13.5, the onset of the ‘secondary transition’ wave of acinar and ß-cell differentiation (Gittes, 2009; Pictet and Rutter, 1972). Hes1 was initially mosaic throughout the epithelium (Fig. 1B), partly overlapping with the ‘tip cell’ marker carboxypeptidase A1 (Cpa1) (see Fig. S3A in the supplementary material) (Zhou et al., 2007), but became increasingly confined to Cpa1-negative ductal and centroacinar cells at later stages (Fig. 1C-D; Fig. S3B,C in the supplementary material). A similar restriction of Hes1 from tip cells has been seen previously (Esni et al., 2004), and the late ductal localization prefigures its expression in the adult (Miyamoto et al., 2003; Stanger et al., 2005). Antibody specificity was indicated by the lack of staining in Hes1-deficient embryos (generated as described below) (see Fig. S3D,E in the supplementary material). Together, these data unify previous (generated as described below) (see Fig. S3D,E in the complementary material).

In order to follow the fate of Hes1 cells, we engineered a Hes1CreERT2 allele (henceforth, Hes1C2) by replacing most of the coding region with CreERT2, a tamoxifen-inducible recombinase (Feil et al., 1997) (Fig. 1E; Fig. S1 in the supplementary material). Hes1C2/+ animals were viable and fertile, while most Hes1C2/C2 embryos died between E12.5 and E13.5 (data not shown), as described for Hes1 knockouts (Ishibashi et al., 1995).

To confirm that Hes1C2 was active in Hes1-expressing cells, we immunostained for Hes1, Cre and lacZ in E12.5 embryos double-transgenic for Hes1C2 and the lineage reporter R26RlacZ (Soriano, 1999), which had received tamoxifen (TM) by maternal gavage at E9.5. We observed close overlap between Cre and Hes1 (see Fig. S4A-F in the supplementary material), indicating that Hes1C2 recapitulates endogenous Hes1 expression. Furthermore, the lacZ lineage marker was widely expressed in the pancreatic epithelium, including by numerous cells that continue to express Hes1 (see Fig. S4G-I, arrowheads, in the supplementary material). In organ cultures, 4-hydroxytamoxifen treatment of dorsal pancreatic buds from E11.5 embryos double-transgenic for Hes1C2 and the lineage reporter R26RlacZ (Srinivas et al., 2001) induced widespread epithelial EYFP expression. Labeling was almost abolished by pretreatment with γ-secretase inhibitor DAPT (Dovey et al., 2001) (see Fig. S5 in the supplementary material), indicating that Hes1C2 expression requires Notch activity. Together, these results indicate that Hes1C2 is expressed and regulated in the same way as endogenous Hes1, and that Hes1C2 can be used to follow the fate of Notch-responsive cells.

Progressive lineage restriction of embryonic Hes1+ pancreatic progenitor cells

We performed additional crosses between Hes1C2 and R26RlacZ, administered a single 2 mg tamoxifen dose to pregnant females between E9.5 and E15.5, and analyzed lacZ expression in the pancreas and gut at E17.5 (n=3-5 embryos per treatment group). No recombination was detected in the absence of tamoxifen (Fig. 2A,F). The labeling frequency in the pancreas was highest (~25%) when tamoxifen was given at E9.5 (Fig. 2B), and decreased with later treatment: ~12% lacZ+ with TM at E11.5, ~8% at E13.5 and ~5% at E15.5 (Fig. 2C-E). In this and other experiments, we analyzed multiple sections spaced throughout the specimen, to avoid errors due to stochastic variations in labeling efficiency (see Fig. S2 in the supplementary material).] The Hes1C2 labeling pattern agrees with the downregulation of endogenous Hes1 expression (Fig. 1A-D), and was reproduced using the R26RlacZ reporter (data not shown). In contrast to the pancreas, liver labeling by Hes1C2 increased with later tamoxifen administration, particularly in cells adjacent to the portal veins (Fig. 2F-J). Almost all labeled cells in the liver expressed the ductal plate markers CK19 and E-cadherin (see Fig. S6 in the supplementary material and data not shown), consistent with studies showing that Notch and Hes1 promote intrahepatic bile duct development (Antoniou et al., 2009; Geisler et al., 2008; Kodama et al., 2004; Lozier et al., 2008; Zong et al., 2009).

To determine the fate of Hes1C2-labeled cells in the pancreas, we stained these specimens for endocrine and exocrine differentiation markers, and calculated the fraction of labeled (lacZ+) cells expressing each marker. Scoring over 1000 lacZ+ cells in each experimental group (see Table S1 in the supplementary material), we found that Hes1+ cells labeled at E9.5 generate both endocrine and exocrine progeny, with roughly one-third of all lacZ+ cells co-expressing insulin (ß-cells) or glucagon (α-cells), and the remainder comprising amylase+ acinar and DBA+ duct cells (Fig. 3A-D, I-J). [Note that although these counts were derived from images taken on a compound microscope, we obtained essentially identical numbers with images generated by confocal microscopy...]

**Fig. 1. Hes1 expression and targeting.** (A-D) Staining for Hes1 (green) and the epithelial marker E-cadherin (red), in embryonic pancreata of the indicated stages. Scale bar: 100 μm. (E) Schematic of wild-type and targeted Hes1. The wild-type Hes1 locus is depicted at the top (exons boxed and numbered, UTRs in grey and coding regions in black), for comparison with Hes1C2, in which the CreERT2 gene and bovine growth hormone polyadenylation signal (pA) are placed in-frame with the Hes1 start codon, replacing much of the ORF.
This finding implies that early Hes1+ cells are multipotent, a conclusion supported by a low-dose clonal labeling approach, previously used to demonstrate tip cell multipotency (Zhou et al., 2007) (see Fig. S7 in the supplementary material). Together, these results are consistent with Notch signaling through Hes1 to maintain early multipotent progenitors.

Later Hes1+ cells continued to generate exocrine progeny, while appearing to lose endocrine differentiation capacity: almost no α-cells were labeled by TM treatment at E13.5, and β-cell labeling approached zero at E15.5 (Fig. 3E-J). Reduced islet contribution was also seen when comparing E13.5-labeled pancreata, 2 weeks after birth, with those labeled at E9.5 (Fig. 3K-N). Delivery of live pups in these experiments required lower tamoxifen doses, which resulted in decreased labeling overall but did not affect the distribution of labeled cells among differentiated lineages. The fact that labeling efficiency was uncoupled from label distribution implies that Hes1C2 drives recombination within a cell population of relatively homogeneous potential for endocrine, duct and acinar differentiation, with the proportion of labeled cells depending on TM dose. We cannot exclude the existence of cells expressing Hes1 at levels too low for labeling by Hes1C2, the developmental potential of which might differ from those observed here. Nonetheless, our results suggest that Hes1 expression shifts from multipotent to exocrine-restricted progenitors (Fig. 3O), consistent with Hes1 having to turn off before the pro-endocrine gene Neurog3 can turn on (Jensen et al., 2000; Lee et al., 2001).

Ectopic Notch activation blocks differentiation of early but not late Hes1+ cells

The wave of acinar and islet cell differentiation that occurs at the secondary transition coincides with Hes1 downregulation (Fig. 1A-D), and we have previously shown that artificially preventing Notch downregulation blocks this differentiation process (Murtaugh et al., 2003). This was achieved by crossing the pan-pancreatic driver Pdx1Cre (Gu et al., 2002) to Rosa26<sup>NothalIC-BRES-GFP</sup> (henceforth, Rosa26NIC), which drives co-expression of activated mouse Notch1 and GFP (Murtaugh et al., 2003) (Fig. 4A,G). To determine whether early and late Hes1+ cells are similarly susceptible to Notch, we crossed Hes1<sup>C2+</sup> to Rosa26NIC.

Pregnant females received a single 2 mg TM dose between E9.5 and E15.5, and double-transgenic offspring were analyzed at E17.5 (n=2 or 3 per timepoint, see Table S1 in the supplementary material). After TM at E9.5 or E11.5, GFP+ cells (expressing activated Notch) formed cystic structures lacking endocrine or acinar marker expression (Fig. 4B-C,H-I). This phenotype resembled that obtained with Pdx1Cre (Fig. 4A,G), and agrees with early Hes1+ cells representing multipotent, Notch-sensitive progenitors. The GFP+ epithelia stained with DBA lectin, which marks mature ducts as well as embryonic progenitors (Kobayashi et al., 2002), but their cystic morphology distinguished them from the narrow and highly-branched ducts normally present at these stages (Fig. 4G-K).

Although Hes1<sup>C2+</sup> labeled very few islet cells at E13.5 or E15.5 (Fig. 3I), Rosa26NIC prevented even this low level of islet differentiation (Fig. 4D,E). By contrast, exocrine differentiation of E13.5-E15.5 Hes1+ cells appeared to be partially Notch resistant, as GFP+ cells were found integrated into normal acini and ducts (Fig. 4J-K). To determine if the blunted effects of late Rosa26NIC activation were secondary to the overall decrease in Hes1C2 labeling efficiency (Fig. 2), we repeated E9.5 treatment with a lower tamoxifen dose (0.5 mg), to activate fewer cells. As previously, the rare GFP+ cells observed in this experiment formed abnormal cystic tubules (Fig. 4F,L), suggesting that early Notch activation can disrupt exocrine differentiation without a ‘critical mass’ of affected cells.

When Rosa26NIC is activated by Pdx1Cre, all cells exhibit a Pdx1<sup>high</sup> ‘trapped progenitor’ phenotype (Murthaugh et al., 2003) (see Fig. S8A,D in the supplementary material). When Notch was activated by Hes1<sup>C2+</sup> at E15.5, GFP+ cells were negative for Pdx1, which instead was expressed only by β-cells (see Fig. S8C,F in the supplementary material). Cells in which Rosa26NIC was induced at E9.5 exhibited modest Pdx1 upregulation (see Fig. S8B,E in the supplementary material), suggesting that they retained a partial progenitor-like identity (Fig. 4N).

Further analysis of late-induced Hes1<sup>C2+</sup>; Rosa26NIC+ pancreata revealed that Notch activation at E13.5 caused most GFP+ cells to adopt a ductal rather than acinar fate, whereas after E15.5 activation the proportions were reversed (Fig. 4M). The latter resembles the wild-type distribution observed with R26RlacZ (Fig. 3J), suggesting that Notch activation at E15.5
does not perturb exocrine differentiation, while E13.5 activation drives bipotent progenitors toward a duct fate (Fig. 4N). Activated Notch can also respecify endocrine precursors to ducts (Greenwood et al., 2007), and this pro-ductal activity may underlie the pathological effects of Notch in pancreatic cancer (De La O et al., 2008).

**Lineage tracing Hes1+ cells in the adult pancreas and intestine**

The question of whether pancreatic progenitor cells persist after birth is a matter of long-standing controversy, particularly with respect to adult differentiation of β-cells. Although several lineage-tracing studies indicate that adult duct and acinar cells do not generate new β-cells in the resting or regenerating pancreas (Desai et al., 2007; Kopinke and Murtaugh, 2010; Solar et al., 2009), these have not excluded the existence of specialized progenitor cells. Centroacinar cells (CACs), in particular, have been proposed to behave as β-cell progenitors (Hayashi et al., 2003; Nagasao et al., 2003; Rovira et al., 2010). CACs express higher levels of Hes1 than do other exocrine cells (Miyamoto et al., 2003; Stanger et al., 2005), and we used Hes1C2 to determine whether these or other Hes1+ cells behave as adult stem or progenitor-like cells.

To identify Hes1-expressing cells in the adult pancreas, we administered 10 mg tamoxifen to 2-month-old (P60) Hes1C2/+; R26REYFP/+ mice (n=4), and analyzed EYFP expression ~48 hours later. Consistent with prior studies of Hes1 expression, we found EYFP labeling of approx. one-quarter of CK19+ centroacinar cells (Fig. 5A), as well as a lesser proportion of labeled cells within larger ducts (Fig. 5B). We also observed a small fraction of EYFP+ acinar cells, suggesting Hes1 expression by rare, differentiated acinar cells (Fig. 5C). To follow the longer-term fate of adult (P60) Hes1+ cells, we compared quantitatively the labeling obtained at 7 days post-TM,
reflecting the initial differentiation state of Hes1+ cells, to that observed after a 2-month ‘chase’, in which time cells might have adopted new fates. As in the 48-hour chase experiment, we detected acinar, duct and CAC labeling after 7 days, which persists at 2 months (Fig. 5D-G). Scoring the labeling index of each differentiated cell type (n=3-10 mice analyzed per condition) (see Table S2 in the supplementary material), we found that ~1% of acinar cells were EYFP+ at each timepoint [1.4±0.3% (s.e.m.) at 7 days, 1.3±0.3% at 2 months; Fig. 5H]. By contrast, the labeling index of duct cells (defined here as CK19+ epithelial cells not embedded within acini) increased by roughly twofold, from 8.0±0.6% EYFP+ at 7 days to 15.5±0.6% EYFP+ after 2 months (Fig. 5H). This might indicate that Hes1 marks a subpopulation of proliferating duct cells, consistent with a mitogenic role for Notch in this lineage (Golson et al., 2009).

Regarding centroacinar cells specifically, we found a similar labeling index at both timepoints (27.0±2.9% EYFP+ at 7 days, 26.5±0.9% EYFP+ at 2 months). The relative labeling indices of ducts and CACs raises an alternative explanation of why duct labeling increases over time, namely that expansion of the ductal tree is driven by descent from CACs. Although anatomically plausible, this hypothesis requires work beyond the scope of this study.

Unexpectedly, we also detected Hes1C2-labeled cells within islets. In islets and throughout the pancreas and other organs, Hes1C2 labeled numerous endothelial cells (~20% in all experiments) (see Fig. S9 in the supplementary material and data not shown), which we have not analyzed further. With respect to endocrine cells, we did not observe a single Hes1C2-labeled β-cell in these experiments, out of over 2000 insulin+ cells scored at each timepoint (see Table S2 and Fig. S9A-B in the supplementary material). We did find rare glucagon+ α-cells marked by Hes1C2 at both timepoints (3.2±0.4% EYFP+ at 7 days post-TM, 5.7±1.1% at 2 months) (see Fig. S9C-D in the supplementary material), as well as after very short chase periods (12-24 hours post-TM, data not shown). Although the increased α-cell labeling with time is statistically significant (P<0.05), its biological relevance is unclear: it could indicate rare neogenesis.

Fig. 4. Ectopic Notch activation blocks differentiation of early but not late Hes1+ cells. (A-L) Rosa26NIC was activated by Pdx1Cre or by Hes1C2/+ following TM treatment between E9.5 and E15.5. E17.5 pancreata were stained for co-expression of GFP (green), marking Rosa26NIC expression, with the endocrine markers insulin and glucagon (top, red and white) or exocrine markers DBA and amylase (bottom, red and purple). Rosa26NIC activation in Pdx1+ or E9.5 Hes1+ cells blocks islet and acinar differentiation, and induces DBA+ cysts (A,B,G,H). Lower-dose activation of Hes1C2 at E9.5 induces similar GFP+ cysts (E,L). With later stage activation, GFP+ cells assume an increasingly normal appearance (C,E,I,K), including integration into normal ducts and co-expression of amylase (closed and open arrowheads). Scale bar: 100 µm. (M) Quantitative distribution of GFP+ cells among amylase+ (blue) and DBA+ (red) cells at E17.5, after Rosa26NIC activation at E13.5 or E15.5 (~100 GFP+ cells scored per sample). Broken lines indicate the normal distribution of Hes1C2-labeled cells after TM treatment at these stages (from Fig. 3J). Notch activation at E13.5 promotes duct development, whereas activation at E15.5 does not perturb normal exocrine differentiation of Hes1+ cells. Results are means±s.e.m. (N) Notch activation in early (E9.5-E11.5) Hes1+ progenitor cells (light orange) prevents normal differentiation and induces a progenitor/duct-like phenotype. At E13.5, Notch promotes mature duct development, whereas activation at E15.5 does not affect differentiation.
representing approx. two-thirds of all EYFP+ cells in the intestinal above the crypts (Fig. 6A,B). The location of the former cells, 12 hours, of single cells in the crypt base region as well as just also found that EYFP, suggesting labeling of intestinal stem cells (Fig. 6C,D). We regimen appeared to capture most behave as adult precursors for this cell type. Our TM treatment (P60) mark stem cells. Low dose (2 mg) tamoxifen treatment of adult (2 mg) tamoxifen treatment of adult 

...EYFP- cells in the adult pancreas; assuming a similar dose-response relationship in the... 

...of duct cells increases. Acinar and CAC labeling does not change over time, whereas... 

**Fig. 5. Hes1 expression and lineage tracing in the adult exocrine pancreas.** Adult (P60) Hes1C2/+; R26R<sup>ERT2</sup> mice were treated with tamoxifen and analyzed for EYFP expression (green) after 2-60 days. (A) After short-term labeling, EYFP is expressed by numerous CK19<sup>+</sup> (red)/E-cadherin<sup>+</sup> (white) centroacinar cells (cac). Right, single-channel EYFP and CK19 staining. (B,C) After short-term labeling, EYFP is also detected in CK19<sup>+</sup> duct (du) cells (B, red) and amylase<sup>+</sup> acinar (ac) cells (C, red). (D,E) A similar fraction of labeled acinar (white arrowheads) and centroacinar cells (open arrowheads) is seen after 7-60-day chase periods. (F,G) Between 7 and 60 days post-TM, the fraction of EYFP-labeled duct cells (arrowheads) appears to expand. Scale bars: 50 μm in A-C, 100 μm in D-G. (H) Quantifying labeled cells as a fraction of all acinar (red triangles), duct (blue circles) or centroacinar cells (green squares). Each point represents the labeling index of at least five fields from a single pancreas; mean labeling indices (across multiple pancreata) are indicated by horizontal lines. Acinar and CAC labeling does not change over time, whereas that of duct cells increases. P-values are determined by Tukey’s HSD test.

**Hes1<sup>C2</sup>-labeled cells do not contribute to β-cells after injury**

Although β-cell neogenesis may not be required in the healthy adult pancreas, it might be induced by injury, as in the case of pancreatic duct ligation (PDL) (Inada et al., 2008; Xu et al., 2008). PDL causes inflammation and acinar cell apoptosis distal to the ligation site (Scoggin et al., 2000; Watanabe et al., 1995), which is accompanied by a local increase in β-cell numbers and apparent reappearance of Neurog3<sup>+</sup> β-cell precursors (Wang et al., 1995; Xu et al., 2008). We therefore sought to determine whether Hes1<sup>C2</sup> marks cells capable of β-cell neogenesis in this model.

Ligation of the dorsal (splenic) pancreas lobe was performed as described by others (Scoggin et al., 2000; Solar et al., 2009; Watanabe et al., 1995; Xu et al., 2008); a full description of our observations in this model will be submitted elsewhere. At 7 days after surgery, acinar cells in the ligated region were completely replaced by fibro-inflammatory cells and epithelial tubules...
centroacinar cells in vitro (Rovira et al., 2010), or from negative cells in vivo, they agree with an independent finding that progenitor-like cells upregulating EYFP+/insulin+ cells (encompassing ~10,000 insulin+ cells scanned material), manually scanning the entire area of each section for throughout the ligated region (see Fig. S2 in the supplementary material). From each mouse, we analyzed 8-10 sections spaced evenly neogenesis (Solar et al., 2009; Wang et al., 1995; Xu et al., 2008).

Doubling of $\beta$-cell mass at this stage, possibly owing to ductal data not shown). We did observe widespread labeling of E-cell in any pancreas, regardless of labeling strategy (Fig. 7E,G and data not shown). To follow $\beta$-cells, we administered TM to $\beta$-cell progenitors in the adult. Although these findings do not exclude the possibility of $\beta$-cell differentiation from cultured centroacinar cells in vitro (Rovira et al., 2010), or from $\beta$-negative cells in vivo, they agree with an independent finding that ducts do not generate $\beta$-cells after PDL (Solar et al., 2009).

**DISCUSSION**

$\beta$-cells after PDL (Herrera et al., 1991; Pictet and Rutter, 1972). $\beta$-cell progenitors in the adult. Although these findings do not exclude the possibility of $\beta$-cell differentiation from cultured centroacinar cells in vitro (Rovira et al., 2010), or from $\beta$-negative cells in vivo, they agree with an independent finding that ducts do not generate $\beta$-cells after PDL (Solar et al., 2009).

**Hes1 expression marks intestinal crypt stem cells.**

(A-D) Adult (P60) Hes1C2/+; R26REYFP+ mice received a single tamoxifen dose, and were stained for labeling of the ileum epithelium (EYFP, green; E-cadherin, red) after 12 hours or 30 days. Short-term labeling (A,B) marks cells in the basal crypt (closed arrowhead) and at the crypt-villus junction (open arrowheads). After a 30-day chase (C,D), labeling encompasses the entire crypt-villus unit, indicating stem cell labeling. (E,F) P60 Hes1C2/+; Ctnnb1lox(ex3)/+ mice were left untreated (E) or administered a single tamoxifen dose (F), and analyzed after 45 days. Hematoxylin and Eosin staining reveals normal morphology of untreated intestine, whereas the small intestines of TM-treated mice exhibit numerous microadenomas (arrowheads) accompanying general tissue disorganization. Outlines indicate areas stained for Ki67 on adjacent sections, which reveal TM-induced expansion of the proliferative compartment from crypts (open arrowheads) to more distal epithelium (closed arrowheads). (G-J) Hes1C2/+; R26RLacZ/+ mice received a single 5 mg TM dose at P60 and were chased for 180 days before analysis by whole-mount X-gal staining of specific intestinal segments. Uniformly lacZ+ crypts are detected in all segments, at a decreasing frequency from anterior to posterior. Staining of distal villi was inconsistent (e.g. G) owing to poor penetration of substrate. Scale bars: 100 $\mu$m.

**Hes1 lineage and Notch function in the embryonic pancreas**

Our results confirm and significantly extend prior studies of Notch-responsive cells in the embryonic pancreas. For example, mapping Notch1 receptor activation in vivo, via N1IP-Cre, reveals scattered labeling throughout the exocrine and endocrine pancreas (Vooijs et al., 2007). Although N1IP-Cre identifies the range of cell types that had experienced Notch1 signaling at some prior stage, it cannot determine when that signaling occurred. As our CreERT2 approach allowed us to mark cells expressing Hes1 at specific developmental stages, we could show that most islet-fated cells had received Notch signals only before the ‘secondary transition’, a wave of endoderm differentiation spanning ~E13.5 to birth in the mouse (Herrera et al., 1991; Pictet and Rutter, 1972).
The fact that Hes1 can directly repress Neurog3 (Lee et al., 2001), together with the excessive α-cell differentiation observed in Hes1 mutants (Jensen et al., 2000), might suggest that Hes1 downregulation is rate-limiting for endocrine specification. However, Neurog3+ endocrine precursors continue to be generated throughout the secondary transition, with a peak at E15.5 (Gradwohl et al., 2000; Gu et al., 2002), and the majority of β-cells differentiate between E15.5 and birth (Herrera et al., 1991). Our results suggest that these cells must derive from progenitors that turn on Neurog3 several days after having turned off Hes1, arguing that endocrine specification is not immediately induced upon Notch-Hes1 downregulation. Indeed, the secondary transition appears to proceed normally in Notch1/Notch2 double mutants (Nakhai et al., 2008), suggesting that Notch-independent mechanisms control the timing of Neurog3 expression and islet differentiation in late embryogenesis.

Prior to the secondary transition, both Cpa1+ tip cells and Hnf1b+ ducts contain multipotent progenitors (Solar et al., 2009; Zhou et al., 2007). Hes1 is expressed in and labels both tip cells and ducts during early pancreas development (see Fig. S3A in the supplementary material and data not shown), suggesting that it marks multipotent progenitors regardless of anatomical location. After E13.5, Cpa1+ cells behave as acinar-restricted precursors, whereas Hnf1b+ cells become restricted to islet and duct fates (Solar et al., 2009; Zhou et al., 2007). To reconcile these observations with our hypothesis that Hes1 marks bipotential acinar/duct progenitors after E13.5, we suggest that acinar-restricted Cpa1+ cells derive from bipotential Hes1+ cells from ~E13.5-E15.5. Indeed, analysis of GFP perdurance in Sox9-EGFP transgenic embryos suggests that ducts give rise to acini through at least E14 (Seymour et al., 2008). That late duct-to-acinar differentiation was not observed with Hnf1b-CreERT2 may reflect inefficient labeling by this transgene in utero (Solar et al., 2009), combined with the overall rarity of Hes1+ progenitors at these stages (Figs 1, 2). In zebrafish, Notch is required for duct specification of exocrine-restricted progenitors (Yee et al., 2005), and our results suggest that Notch also promotes duct development in late mouse embryogenesis.

**Hes1 expression and phenotypic plasticity in the adult pancreas**

Notch is implicated in self-renewal of adult stem cells (Chiba, 2006), and Hes1C2 robustly labels stem cells in the intestinal crypts. Under conditions sufficient to label the majority of intestinal stem cells, however, we do not find evidence that Hes1C2 labels stem-like cells in the adult pancreas. Instead, we find that Hes1C2 is active in several mature cell types, of which centroacinar cells are the most highly labeled. Previous studies indicate that Hes1 expression and Notch activity are highest in CACs, and lower in more proximal ductal elements (Miyamoto et al., 2003; Parsons et al., 2009; Stanger et al., 2005), closely paralleling the Hes1C2 labeling pattern. Although we have not obtained reliable Hes1 immunostaining in adult pancreata (data not shown), Hes1C2 labeling suggests that it is also expressed by rare differentiated α-cells and acinar cells. Whether Notch has a functional role in these cells remains to be determined. Importantly, we never observe Hes1C2 labeling of insulin+ β-cells, suggesting little or no contribution to these cells from the adult Hes1 lineage.

This result appears inconsistent with the finding that CACs, isolated based on high aldehyde dehydrogenase activity, can give rise to β-cells and other cell types in vitro (Rovira et al., 2010). Interestingly, however, the cells isolated in that study expressed only low levels of Hes1, suggesting that they represent a distinct subpopulation of CACs. Indeed, we find that only approx. one-quarter of CACs are labeled by Hes1C2 using our standard tamoxifen dose, and that this proportion is not increased by a threefold higher dose. Our results therefore constitute in vivo evidence for heterogeneity within the duct and CAC compartments, and suggest that the Hes1C2 subpopulation does not normally give rise to β-cells.

Alternatively, the failure of Hes1C2 to label β-cells might reflect limitations imposed by the micro-environment of the mature pancreas, e.g. active Notch signaling reinforcing ductal fate, which could be removed in tissue culture or during regeneration. To address this, we adopted an injury model, pancreatic duct ligation, which has provided suggestive evidence of β-cell neogenesis from ductal progenitors (Wang et al., 1995; Xu et al., 2008). In rats and mice, PDL is reported to lead to a local doubling of β-cell mass within 1 week (Wang et al., 1995; Xu et al., 2008), together with inflammation, acinar cell apoptosis and ductal hyperplasia (Scoggin et al., 2000; Watanabe et al., 1995). At 7 days post-PDL, we find that Hes1C2-labeled cells contribute to the abnormal ductal epithelium, but
not to β-cells located either in islets or within or near ducts. We note that identical results were obtained using Hnf1b-CreERT2, which labels cells throughout the ductal network (Solar et al., 2009), suggesting that new β-cells arise after PDL either from pre-existing β-cells, or from a duct subpopulation that expresses neither Hnf1b nor Hes1 (Inada et al., 2008).

Our results do not exclude the possibility that CACs or other Hes1+ cells could give rise to β-cells more than 7 days post-PDL, although further increases in β-cell mass beyond this timepoint have not been reported (Wang et al., 1995; Xu et al., 2008), and adipocyte infiltration at later stages may produce secondary effects on islets (Watanabe et al., 1995). It is also possible that other injury models might evoke β-cell differentiation from Hes1+ cells, much as glucagon+ α-cells can transform into β-cells after extreme β-cell loss, despite an otherwise absolute barrier to interconversion (Thorel et al., 2010). We also note that, as Hes1+ cells label a minority of CACs, our study does not definitively test CAC differentiation potential.

Several other CreERT drivers, particularly those with stringent tamoxifen dependence, have been shown to recombine only a minority of their putative target cells (Desai et al., 2007; Dor et al., 2004; Solar et al., 2009), raising the possibility of unlabeled subpopulations. Our results, however, suggest that Hes1+ does label most Hes1+ cells in the adult, but that Hes1+ duct and centroacinar cells cannot generate β-cells under the conditions studied here. In sum, Hes1+ provides a new tool to test the role of Notch-responsive cells in physiological and pathological conditions, and our studies raise the issue of whether Notch activity functionally subdivides pancreatic ductal cells into those with and without progenitor-like potential.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.053843/-/DC1

References


Lineage-tracing of *Hes* cells

RESEARCH ARTICLE


Lammert, E., Brown, J. and Melton, D. A.


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Table S1. Cell counts for analyses of embryonic lineage distribution

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Timepoint</th>
<th>Markers scored</th>
<th>Number of embryos</th>
<th>Number of fields/embryo</th>
<th>Total number of lineage* cells scored</th>
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For each figure, we list the number of embryos analyzed per timepoint, the number of microscopic fields scored per embryo and the total number of *lineage marker-expressing cells (across all embryos) scored for co-expression of the indicated differentiation markers.
Table S2. Cell counts for calculations of adult labeling indices

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For each figure, we list the number of adult mice analyzed per timepoint, the number of microscopic fields scored per mouse and the total number of *differentiation marker-expressing cells (across all mice) scored for co-expression of the R26R<sup>EYFP</sup> lineage marker. Note that for the duct ligation experiments (Fig. 7), we manually scanned the entire surface of each slide for labeling of insulin* cells, using the specimens scored here for labeling of CK19* and E-cadherin* cells, as well as additional specimens in which CK19* and E-cadherin* cells were not analyzed.
**Figure S1**

A

- **Hes1<sup>wt</sup>**
  - 1 kb
  - Targeting construct

- **Hes1<sup>CFNF</sup>**
  - S N 1 2 3 4 S N

- **Hes1<sup>CF</sup>**

B

- Clone # 38 62 wt
  - NcoI
    - 8.0 kb
    - 6.5 kb
  - SpeI
    - 5.8 kb
    - 3.5 kb

Probe
distribution = $\frac{\text{marker}^+ \text{ LacZ}^+}{\text{total LacZ}^+}$

labeling index = $\frac{\text{marker}^+ \text{ LacZ}^+}{\text{total marker}^+}$

Figure S2
Figure S3
Figure S4
Figure S5
Figure S6
Figure S7
Figure S8
Figure S9