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Notch signaling differentially regulates the cell fate of early endocrine precursor cells and their maturing descendants in the mouse pancreas and intestine

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ABSTRACT

Notch signaling inhibits differentiation of endocrine cells in the pancreas and intestine. In a number of cases, the observed inhibition occurred with Notch activation in multipotential cells, prior to the initiation of endocrine differentiation. It has not been established how direct activation of Notch in endocrine precursor cells affects their subsequent cell fate. Using conditional activation of Notch in cells expressing Neurogenin3 or NeuroD1, we examined the effects of Notch in both organs, on cell fate of early endocrine precursors and maturing endocrine-restricted cells, respectively. Notch did not preclude the differentiation of a limited number of endocrine cells in either organ when activated in Ngn3^{+} precursor cells. In addition, in the pancreas most Ngn3^{+} cells adopted a duct but not acinar cell fate; whereas in intestinal Ngn3^{+} cells, Notch favored enterocyte and goblet cell fates, while selecting against endocrine and Paneth cell differentiation. A small fraction of NeuroD1^{+} cells in the pancreas retain plasticity to respond to Notch, giving rise to intraislet ductules as well as cells with no detectable pancreatic lineage markers that appear to have limited ultrastructural features of both endocrine and duct cells. These results suggest that Notch directly regulates cell fate decisions in multipotential early endocrine precursor cells. Some maturing endocrine-restricted NeuroD1^{+} cells in the pancreas switch to the duct lineage in response to Notch, indicating previously unappreciated plasticity at such a late stage of endocrine differentiation.

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Introduction

Endocrine cells in the pancreas and intestine differentiate from multipotential epithelial cells derived from the early gut endoderm. In the pancreas, relatively undifferentiated epithelial cells give rise to the duct, acinar, and endocrine lineages prior to birth. At least five different endocrine cell types form the islets of Langerhans including insulin producing β cells, as well as α, ∂, PP, and ε cells that produce glucagon, somatostatin, PP, and ghrelin (Habener et al., 2005; Oliver-Krasinski and Stoffers, 2008). In contrast, enteroendocrine cells that express one or more of 12 hormones continuously differentiate from precursors throughout postnatal life.

Endocrine differentiation in both the pancreas and intestine is regulated by the temporal expression of basic helix loop helix (bHLH) transcription factors to sequentially restrict subsequent differentiation to specific lineages. Expression of the bHLH transcription factor, Neurogenin 3 (Ngn3) initiates endocrine differentiation following specification of the pancreatic epithelium by the homeodomain protein, Pdx1 early in pancreagenesis (Gu et al., 2002).

The absence of pancreatic endocrine cells in Ngn3$^{-/-}$ mice suggests that Ngn3 is required for their specification (Gradwohl et al., 2000). Lineage analysis of the descendants of Ngn3^{+} cells showed that all endocrine cells in the pancreas arose from Ngn3^{+} cells, indicating that the effects of Ngn3 were cell autonomous. However, lineage tracing also revealed that small numbers of acinar and duct cells arose from Ngn3 expressing cells, suggesting that Ngn3^{+} cells were not restricted to an endocrine cell fate (Gu et al., 2003; Schonhoff et al., 2004).

NeuroD1, another bHLH protein, was initially described as an activator of the insulin gene (Naya et al., 1995). NeuroD1 knockout mice develop severe diabetes with reduced numbers of β cells (Naya et al., 1997). The absence of NeuroD1 in Ngn3 null mice

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indicates that NeuroD1 is downstream of Ngn3 (Gradwohl et al., 2000). Ngn3 and the homeodomain protein, NKX2.2, (Anderson et al., 2009; Huang et al., 2000) directly activate NeuroD1 transcription, suggesting that NeuroD1 is expressed at a later stage of islet differentiation.

In the intestine, the three secretory lineages, enteroendocrine, Paneth, and goblet cells require the bHLH protein, Atoh1 for differentiation (Shroyer et al., 2005; Yang et al., 2001). Presumably, Ngn3 initiates endocrine differentiation as enteroendocrine precursor cells segregate from a common secretory progenitor cell. As in the pancreas, intestinal enteroendocrine cells are absent from Ngn3 null mice although some endocrine cells in the stomach differentiate in the absence of Ngn3 expression (Jenny et al., 2002; Lee et al., 2002). Much like the pancreas, Ngn3 expressing cells in the intestine are multipotential, contributing to the goblet and Paneth cell lineages (Schonhoff et al., 2004). Secretin and cholecystokinin cells fail to develop in neuroD1 null mice whereas other enteroendocrine cell types are present (Naya et al., 1997). NeuroD1 is expressed in nearly all enteroendocrine cell types where it may have a role in inhibiting cell proliferation as cells mature (Mutoh et al., 1998; Ratineau et al., 2002).

A number of studies suggest that Notch signaling inhibits endocrine differentiation in both the pancreas and the intestine. Many of the effects of Notch result from its inhibition of bHLH proteins that activate cellular differentiation programs. Thus Ngn3 and NeuroD1 are potential targets of Notch in the pancreas and the intestine. Notch signaling increases expression of its transcriptional effector protein, Hes1, in the pancreas (Jarriault et al., 1998), inhibiting expression and/or transcriptional activity of bHLH proteins (Davis and Turner, 2001). The Ngn3 gene has multiple Hes1 binding sites and is repressed by Hes1, suggesting it may be a target of Notch (Lee et al., 2001).

Activation of Notch at a relatively early stage of pancreatic development in cells expressing Pdx1 resulted in failure to develop both endocrine and acinar cells with the remaining pancreatic cells trapped in a relatively undifferentiated state (Hald et al., 2003; Murtaugh et al., 2003). Likewise, activation of Notch in cells expressing either villin or fatty acid binding protein showed a significant reduction in enteroendocrine and goblet cells (Fre et al., 2005; Stanger et al., 2005). The timing and context of Notch activation is a major determinant of its effects on organogenesis and differentiation. The studies described above involved the effects of Notch activation in broad populations of multipotential cells in each organ that give rise to several cell lineages in addition to endocrine cells. The observed effects of Notch in these studies were likely the result of its activation prior to the initiation of endocrine differentiation. The inhibitory role of Notch was further suggested by excessive endocrine differentiation seen with widespread disruption of Notch function in the pancreas (Apelqvist et al., 1999; Jensen et al., 2000) and in the fetal intestine (Jensen et al., 2000).

The effects of Notch activation in endocrine precursor cells in the pancreas have been examined in limited detail. The results were interpreted to suggest complete inhibition of endocrine differentiation in early endocrine precursors with less pronounced effects in maturing islet cells (Greenwood et al., 2007; Murtaugh et al., 2003). The effects of Notch activation in enteroendocrine precursor cells have not been described.

In the present study, we have examined the effect of conditionally activating Notch signaling in early and late endocrine precursor cells expressing Ngn3 or NeuroD1, respectively in the pancreas and intestine. Unexpectedly, activation of Notch in $Ngn3^{+}$ cells did not prevent the initiation of endocrine differentiation in the pancreas or the intestine. However a number of islet cell types failed to mature in the pancreas. Many cells switched to a duct cell fate in pancreas and enterocyte and goblet cell fate in intestine. Surprisingly, some $NeuroD1^{+}$ cells in the pancreas respond to Notch and differentiate into duct cells, whereas maturing enteroendocrine precursors were relatively unaffected by Notch.

Material and methods

Animals

Sequences encoding Cre recombinase that included a nuclear localization signal and simian virus 40 polyadenylation sequences were introduced into a murine BAC clone, RPCI-23188B11 (Invitrogen) containing the NeuroD1 locus, by homologous recombination in E. Coli with a linear DNA fragment amplified from the suicide vector pKD4 as described previously (Cotta-De-Almeida et al., 2003; Datsenko and Wanner, 2000; Schonhoff et al., 2004). The Cre encoding sequences were inserted into the translation initiation ATG of the NeuroD gene. A *Ngn3-CreERT2* transgene was generated by inserting CreERT2 sequences into the same Ngn3 BAC described previously (Schonhoff et al., 2004).

Transgenic mice were generated by pronuclear injection of the purified circular *NeuroD1-Cre* BAC or *Ngn3-CreERT2* BAC DNA into the pronuclei of fertilized oocytes of B6XB6D2F1 mice. Founders were generated and were identified by genotyping with primers specific for the Cre transgene. We generated 2 *NeuroD1-Cre* and 3 *Ngn3-CreERT2* pedigrees. For each line, the pedigrees showed nearly identical pattern of Cre expression. The Ngn3-Cre mice have been described earlier (Schonhoff et al., 2004). The PCR primers used for generating BAC *NeuroD1-Cre* transgene were: Sense- TGCTTGCCTCTCTCCCTGTTCAATACAGGAAGTGGAAACATGCCCAAGAAGA AGAGGAA and antisense-GGCTCGCCCATCAGCCCGCTCTCGCTGTATGATTTGGTCATCCTCCTTAGTTCCTATTCCGA.

For lineage tracing experiments, *NeuroD1-Cre* transgenic mice were crossed with *ROSA26-LacZ* (B6.129-Gt26Sor^{tm}) or *ROSA26-EYFP* (B6.129 × 1-Gt (ROSA) 26Sortm1(EYFP) Cos) indicator mice (Soriano, 1999). To determine if Ngn3 and NeuroD1 expressing cells are susceptible to Notch, Ngn3-Cre (Schonhoff et al., 2004), *NeuroD1-Cre* and *Ngn3-creERT2* mice were crossed with *ROSA26* $^{Notch/Notch}$ knock-in mice that contain the intracellular domain of murine Notch1 and a bicistronic nuclear EGFP reporter downstream of a floxed stop sequence (Murtaugh et al., 2003). Cre activity was induced in *Ngn3-creERT2;ROSA*$^{Notch/+}$ mice by treating 6 weeks old mice with 2 mg tamoxifen (Sigma, T5648) dissolved in corn oil with one intraperitoneal injection per day for 5 days. Tissues were harvested and analyzed 7 day later. The Institutional Animal Care and Use Committee approved all vertebrate animal studies in accordance with NIH regulations.

Immunohistochemistry

Tissues for X-Gal staining were performed as previously described (Schonhoff et al., 2004). Fixed tissue samples were processed for either frozen or paraffin sections using standard methods.

The following primary antibodies were used for immunohistochemical and immunofluorescence analysis: Goat anti-NeuroD1 (1:500, Santa Cruz), Goat anti-EGFP (1:100, Abcam), Rabbit anti-GFP (1:10,000, Invitrogen), Guinea pig anti-Insulin (1: 5000, Sorin Biomedica), Guinea pig anti-PYY (1: 5000, a gift from G. Aponte, University of California, Berkley), Rabbit anti-ChromograninA (1:1000, Immunostar), Rabbit anti-Somatostatin (1:8000, a gift from R. Lechan, Tufts Medical Center), Rabbit anti-Glucagon (1: 3000, a gift from M. Appel, University of Massachusetts), Rabbit anti-Pancreatic Polypeptide (1: 5000, a gift from Chance, Eli Lilly), Rabbit anti-β-Gal (1:600, Cappel), Wisteria Fluoribunda Agglutinin (WFA, 1: 600, Sigma), Biotinylated Dolichos Biflorus Agglutinin (DBA, 1:1000, Vector Laboratories), Mouse anti-Neurogenin3

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