Preservation of proliferating pancreatic progenitor cells by Delta-Notch signaling in the embryonic chicken pancreas
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Abstract

Background: Genetic studies have shown that formation of pancreatic endocrine cells in mice is dependent on the cell autonomous action of the bHLH transcription factor Neurogenin3 and that the extent and timing of endocrine differentiation is controlled by Notch signaling. To further understand the mechanism by which Notch exerts this function, we have investigated pancreatic endocrine development in chicken embryos.

Results: In situ hybridization showed that expression of Notch signaling components and pro-endocrine bHLH factors is conserved to a large degree between chicken and mouse. Cell autonomous inhibition of Notch signal reception results in significantly increased endocrine differentiation demonstrating that these early progenitors are prevented from differentiating by ongoing Notch signaling. Conversely, activated Notch1 induces Hes5-1 expression and prevents endocrine development. Notably, activated Notch also prevents Ngn3-mediated induction of a number of downstream targets including NeuroD, Hes6-1, and MyT1 suggesting that Notch may act to inhibit both Ngn3 gene expression and protein function. Activated Notch1 could also block endocrine development and gene expression induced by NeuroD. Nevertheless, Ngn3- and NeuroD-induced delamination of endodermal cells was insensitive to activated Notch under these conditions. Finally, we show that Myt1 can partially overcome the repressive effect of activated Notch on endocrine gene expression.

Conclusion: We conclude that pancreatic endocrine development in the chicken relies on a conserved bHLH cascade under inhibitory control of Notch signaling. This lays the ground for further studies that take advantage of the ease at which chicken embryos can be manipulated. Our results also demonstrate that Notch can repress Ngn3 and NeuroD protein function and stimulate progenitor proliferation. To determine whether Notch in fact does act in Ngn3-expressing cells in vivo will require further studies relying on conditional mutagenesis. Lastly, our results demonstrate that expression of differentiation markers can be uncoupled from the process of delamination of differentiating cells from the epithelium.

Published: 7 June 2007
BMC Developmental Biology 2007, 7:63 doi:10.1186/1471-213X-7-63
Received: 8 December 2006
Accepted: 7 June 2007
This article is available from: http://www.biomedcentral.com/1471-213X/7/63
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Background

The pancreas is an organ containing both exocrine and endocrine cell populations. The exocrine pancreas consists of acini and ducts that produce and transport enzymes and bicarbonate to the digestive tract. Lineage tracing studies have revealed that both endocrine and exocrine cells are derived from Pdx1-expressing progenitors [1-3]. The endocrine cells are organized in the islets of Langerhans which contain five distinct cell types, each characterized by the production of specific peptide hormones [4-6]. Endocrine cells begin to appear soon after the first morphological signs of pancreas formation which occurs approximately at the 25-somite stage in the mouse and chicken [7]. Endocrine development depends on Neurogenin3 (Ngn3) and is initiated by the onset of Ngn3 expression in a subset of pancreatic progenitor cells [8-11]. All endocrine cells are derived from Ngn3 expressing precursors and the vast majority, if not all, of the Ngn3 expressing cells are committed to the endocrine lineage [1,12]

Several studies have demonstrated that Notch signaling is involved in the development of endocrine cells in the pancreas. Mice harboring mutations in the Notch pathway genesDll1, Rlk1-k, and Hes1 all display precocious and excessive endocrine development as early as E9.5 [10,13]. At this stage there is an increase in the numbers of Ngn3 positive cells in Dll1 and RBP-Jκ mutants, and at E10.5 an increase in endocrine cells. Ngn3 expression was not analyzed in Hes1 mutants but these had increased numbers of glucagon-producing cells at E9.5. Together these studies suggest that Notch signaling prevents endocrine differentiation through a mechanism known as lateral inhibition where the Notch ligand Dll1, expressed in differentiating cells, signals through Notch receptors on adjacent cells thereby keeping them undifferentiated or acquiring a secondary fate. However, Notch signaling may act to regulate differentiation by controlling the proneural genes at the transcriptional level or Notch signaling could act in already committed precursors by inhibiting proneural factors posttranslationally. These are not mutually exclusive mechanisms and indeed both have been proposed [14-16]. Later studies have suggested that Notch signaling not only regulates endocrine specification but also inhibits exocrine differentiation [17,18]. This was further supported by the finding that Hes1, a Notch target gene, is active in exocrine precursors in the mouse pancreas and prevents their terminal differentiation, and that loss of Notch signaling in zebrafish accelerate exocrine differentiation [19]. Subsequently, Jagged mediated Notch signaling has been suggested to mediate a fate choice between exocrine and intrapancreatic duct fate from a common precursor cell in zebrafish [20]. This is most likely different from the situation in mice where duct cell progenitors and exocrine progenitors appear to diverge very early in development between E9.5 and E11.5, prior to expression of Jagged-1 [1,21].

Here we show that the expression of pro-endocrine bHLH factors and Notch pathway components is conserved in the embryonic chicken pancreas and identify Hes6-1 as being expressed in the endocrine lineage in the embryonic pancreas. We demonstrate that inhibition of Notch signaling results in increased endocrine differentiation, and that activated Notch1 (Notch1ICD) blocks endocrine development and maintains proliferation of pancreatic progenitor cells in the embryonic chicken pancreas. We demonstrate that Notch1ICD is able to inhibit Ngn3 activity as it prevents Ngn3 induced endocrine differentiation, visualized by loss of NeuroD, Myt1, Hes6-1, Pax6, βIII-tubulin, and glucagon expression but, remarkably, without affecting delamination of the Ngn3-expressing cells from the gut epithelium. We also show that NeuroD-induced endocrine development is sensitive to inhibition by Notch. Lastly, we show that forced expression of Myt1 partly restores βIII-tubulin expression in NotchICD expressing cells. Together, our results demonstrate that Notch regulation of pancreatic endocrine development is conserved in chicken, and raises a possibility of Notch mediated inhibition of Ngn3 and NeuroD protein activity.

Results

The embryonic chicken pancreas expresses pro-endocrine bHLH genes

Pancreatic endocrine development in the mouse relies on the pro-endocrine genes Ngn3 and NeuroD [1,8-10,22] whose activity is, at least partly, controlled by Hes1 and Notch signaling [10,13,17,18]. To determine if these genes are expressed in the chicken pancreas we performed in situ hybridizations on sections of HH st. 22 [23] (E4) and HH st. 31 (E7) chicken pancreas (Fig. 1). These time-points correspond roughly to E11 and E14 of mouse pancreas development where the epithelium expands and the endocrine and exocrine differentiation peaks, respectively. At HH st. 22, Ngn3 was expressed in a few scattered cells within the epithelium (Fig. 1A) while NeuroD was expressed in clusters of endocrine cells located at the distal part of the dorsal pancreatic bud (Fig. 1B) as determined by co-staining for Nkx6.1, a marker of pancreatic progenitor cells as well as β-cells, and glucagon expressed by the α-cells (not shown). The chicken Hes1 homologue, formerly known as Hairy1 [24], now Hes1 after official nomenclature, was expressed broadly in the pancreatic epithelium at HH st. 22 (Fig. 1C) while Hes6-1 was expressed in scattered cells within the epithelium (Fig. 1D). In the HH st. 31 pancreas, the overall expression patterns were similar to the HH st. 22 pancreas (Fig. 1E–L), but we consistently observed that Hes1 expression levels varied between different regions of the epithelium such
that areas with low levels of Hes1 expression corresponded to regions where Ngn3 and Hes6-1 were expressed (Fig. 1E,G,H). To further define the cell types that expressed the four bHLH genes we used immunohistochemical detection of glucagon, amylase, and Nkx6.1 on sections previously subjected to in situ hybridization. We found that regions expressing high levels of Hes1 (boxed area in Fig. 1G) were devoid of glucagon (data not shown), whereas amylase was readily detectable in Hes1-expressing cells (Fig. 1K). Ngn3 and Hes6-1 were both expressed in pancreatic progenitor cells marked by Nkx6.1 [25,26], but absent from glucagon-producing cells (Fig. 1I). NeuroD expression was restricted to clusters of endocrine cells (including Nkx6.1+ insulin-producing cells) adjacent to the Nkx6.1 positive epithelium (Fig. 1F,I). We also analyzed the expression of Hes6-2 and Hes5-1, -2, and -3 [27], but could not detect any transcripts in the pancreas. Lastly, using whole-mount in situ hybridization on micro-dissected HH st. 22 chicken pancreas we observed expression of Delta1, Notch1, and the chicken Jagged-1 and -2 homologs Serrate-1 and -2 (data not shown), but due to technical limitations we were not able to further define the cell types that expressed these genes.

**Forced expression of Notch ligands induces endocrine differentiation**

Expression of pro-endocrine and Notch pathway genes suggests that Notch signaling might regulate endocrine development in the chicken pancreas. If the function of Delta1 is to signal to adjacent progenitor cells and prevent them from entering the endocrine differentiation program, then forced widespread expression of Delta1 in the pancreatic epithelium should reduce the number of endocrine cells formed. To test this prediction, we introduced plasmids expressing Delta1 upstream of an IRES-GFP cassette into the endoderm of HH st. 10–12 and HH st. 13–15 embryos by in ovo endoderm electroporation [11] and assayed pancreatic endocrine differentiation after 72 hours (at HH st. 26–27) by staining for GFP, insulin, and glucagon (Fig. 2). Embryos electroporated with an IRES-GFP vector lacking the insert served as controls. We found that extensive expression of Delta1 in both HH st. 10–12 and HH st. 13–15 embryos resulted in a marked increase in the fraction of GFP-expressing cells that differentiate into endocrine cells, compared to controls (Fig. 2A,B and 2E). At a first glance this result may seem paradoxical, but we speculate that a cell autonomous inhibition of Notch signal reception.

Such embryos showed an increase in endocrine differentiation that was comparable to that seen with Delta1 regardless of whether full-length or truncated Serrate1 was used (Fig. 2, compare C, D to A, B), arguing that the differentiation inducing effects seen when over-expressing full-length Delta1 and Serrate1 is the result of a cell autonomous inhibition of Notch signal reception.
Similar experiments using a truncated Delta1 construct (Delta1-d1, [28]) were inconclusive as very few GFP+ cells remained 48 hours after electroporation and none were detectable after 72 hours. Quantitatively, mis-expression of Delta1 beginning at HH st. 10–12 or HH st. 13–15 resulted in an increase of the fraction of endocrine GFP+ cells from ~40% to ~75% and from ~20% to ~50%, respectively (Fig. 2E). In extreme cases pancreas morphology was severely altered with the dorsal pancreas adopting a tubular morphology with the epithelium composed almost exclusively of endocrine cells (Fig. 3 compare B to A). Notably, forced expression of Delta1 had no endocrine-specific effect in the prospective duodenum (Fig. 3B). If the effect of the ligands were a cell-autonomous inhibition of Notch signaling then this effect should be blocked by a constitutive activation of the Notch pathway in the same cell. To test this, we co-expressed Delta1 with a constitutively active form of Notch1 (Notch1ICD) [17] in the chick pancreas by in ovo electroporation. In agreement with this model we found that Notch1ICD was able to reverse the effect of Delta on endocrine differentiation such that most transfected cells were excluded from the endocrine area under these conditions (Fig. 3B, C).

To test if ectopic expression of the ligands also induced exocrine differentiation we compared the expression of the exo- and endocrine markers amylase and glucagon, respectively, in control and Delta1 electroporated chicken pancreata (Fig 3D, E). GFP-expressing cells were evenly distributed throughout the pancreas epithelium, including exocrine and endocrine cells in the control pancreas. In contrast, the majority of the GFP positive cells expressed glucagon and were excluded from the exocrine cells in Delta1 electroporated pancreata. Furthermore, the Delta1-expressing pancreata were consistently smaller, suggesting that Delta1 induces precocious endocrine differentiation at the expense of progenitor expansion and later born cell types such as the exocrine.

**Constitutively active Notch1 prevents endocrine differentiation and maintains proliferation**

The results above suggest that Notch signaling inhibits endocrine differentiation in the chicken pancreas and that this inhibitory signal must be terminated before differentiation can occur. To test the effect of a constitutive activation of Notch signaling on normal pancreas development we introduced Notch1ICD [17] by in ovo endoderm electroporation at HH st. 13–15 and analyzed endocrine development after 52 hours (HH st. 21–23)(Fig. 4). We found that GFP+ cells in embryos expressing the control vector readily contributed to α- and β-cells as determined by co-localization of GFP with glucagon and insulin (Fig. 4A), while GFP+ cells in Notch1ICD-expressing embryos were only rarely found to co-express glucagon or insulin (Fig. 4D). Quantitative analysis revealed that only ~1% of the GFP+ cells in the Notch1ICD-expressing pancreas were endocrine compared to 8% for control embryos (Fig. 4G), demonstrating that Notch1ICD blocks endocrine differentiation in the chicken pancreas. The few cells we did find co-expressing Notch1ICD and endocrine markers can be explained by the occasional electroporation of existing endocrine cells at the time of electroporation [11].

In addition to reduced endocrine development we also observed that the morphology of the pancreas anlage in Notch1ICD-expressing embryos was disturbed compared to control embryos, such that the Nkx6.1+ pancreas epithelium appeared expanded and highly folded compared to controls (Fig. 4, compare B and E). We hypothesized that the folded epithelium we observed in Notch1ICD-expressing pancreas could be the result of increased proliferation. We therefore analyzed proliferation of the Nkx6.1+ pancreas epithelium using a monoclonal antibody against MPM2 which is a marker for mitotic cells [34]. We found that the fraction of Notch1ICD-expressing cells in the pancreas epithelium that expressed MPM2 was 2.5-fold higher than for corresponding cells expressing the control plasmid (Fig. 4C, D and Fig. 5I), demonstrating that active Notch signaling inhibits differentiation which is coincident with ongoing proliferation of pancreatic epithelial cells. These data lend further support to the notion of Notch signaling acting primarily to maintain a progen-
itor state, rather than inducing an alternative differentiated cell fate [35].

Ngn3 induced cell cycle withdrawal and differentiation is antagonized by Notch1ICD

The results described above suggest that Notch mediated control of endocrine pancreas development is conserved in chicken. Previous results obtained in mice have indicated that Notch signaling acts to repress Ngn3 expression and thereby prevents endocrine differentiation [10,17,18]. However, it is also possible that Notch signaling may additionally act in cells that already have initiated Ngn3 expression by antagonizing the function of the encoded protein Ngn3 [16]. To test if activated Notch could interfere with the ability of Ngn3 to induce endocrine differentiation we took advantage of the ability of ectopic Ngn3 to induce endocrine differentiation in the chick endoderm [11]. We confirmed that Ngn3 induced endocrine differentiation in the chick pancreas (Fig. 5). Most Ngn3 positive cells were Nkx6.1 negative and Pax6 positive, suggesting they had differentiated into α-cells

(Fig. 5A,B). In agreement with this we found that many Ngn3 positive cells expressed glucagon while insulin-expressing cells were rare (Fig. 5C). Mitotic cells were extremely rare among the Ngn3-expressing cells as judged by MPM2 immunoreactivity (Fig. 5D) similar to the situation in mouse where early endocrine cells are post mitotic [22]. To test if active Notch signaling can prevent Ngn3-induced endocrine differentiation in the chick pancreas, we co-electroporated HH st. 13–15 embryos with Ngn3 and Notch1ICD expression vectors and assayed endocrine development after 52 hours. In contrast to embryos electroporated with the Ngn3 vector alone we found that most co-electroporated cells maintained Nkx6.1 expression (Fig. 5E) and displayed a high percentage of MPM2 expression (Fig. 5H,I) whereas very few cells expressed Pax6, glucagon or insulin (Fig. 5F,G). This result demonstrates that activated Notch1 can inhibit the function of the Ngn3 protein.

Hes5-1 and Hes6-1 is inversely regulated by Ngn3 and Notch1ICD

To begin to unravel the mechanism behind Notch-mediated inhibition of endocrine development, we next investigated the expression pattern of Hes1, Hes5-1, Hes6-1, and NeuroD in the pancreas after introduction of Ngn3, Notch1ICD and Ngn3 + Notch1ICD or control vector (Fig. 6). The expression pattern of these genes was not affected in embryos electroporated with control vector (compare
Conversely, Ngn3 electroporation led to loss of Hes1 expression and a robust induction of NeuroD and Hes6-1 in the electroporated area (Fig. 6G,I,J). In contrast, Notch1ICD repressed NeuroD and Hes6-1 expression in the electroporated area (Fig. 6N,O). Notably, we found no evidence to suggest that Notch1ICD up-regulated Hes1 (Fig. 6L) and it even appeared to be down-regulated in the transfected area. Instead, Hes5-1, another Notch target gene [27,36] not normally expressed in the pancreas epithelium, was strongly induced in Notch1ICD expressing cells (Fig. 6M). In the developing nervous system of the mouse, Hes1 and Hes5 are expressed in complementary patterns and Hes5 expression is expanded in the Hes1 null mice and vice versa indicating mutual cross repression [37]. Hes5 expression is also induced in the endoderm of Hes1 null mice [13]. In the chick embryo Hes5-1 is known to repress the expression of other chicken Hes genes [27] and it is possible that it also represses Hes1 under these conditions. Similar results were recently reported by Matsuda et al. who found that ectopic expression of Notch1ICD in chick embryonic stomach endoderm failed to up-regulate transcription of Hes1 and Hairy2, two Hes homologues normally found to be expressed in this tissue [38]. When Ngn3 and Notch1ICD were co-electroporated we observed a robust induction of Hes5-1 expression while Ngn3-induced NeuroD and Hes6-1 expression was lost (Fig. 6Q–T). Hes1 also appeared down-regulated under these conditions.

**Activated Notch1 inhibits Ngn3 and NeuroD induced endocrine differentiation but not delamination**

Our finding that Notch1ICD inhibits both expression and function of Ngn3 is consistent with previous reports showing that X-NotchICD inhibits both the expression and function of the Ngn3 related X-NGNR-1 during Xenopus neurogenesis [39,40]. Furthermore, since X-NGNR-1 can induce XNeuroD expression while the converse is not the case [39] it appears that a unidirectional bHLH cascade regulates neural determination and differentiation (regulated by Neurogenin and NeuroD, respectively). Whether either or both of these processes are repressed by Notch signaling is not fully understood. Furthermore, it is still

**bHLH genes are inversely regulated by Ngn3 and Notch1ICD**

(A-T). In situ hybridization on serial sections from embryonic pancreata 52 hours after electroporation with control vector (A-E), Ngn3 (F-J), Notch1ICD (K-O), or Ngn3 and Notch1ICD (P-T). The expression patterns of Hes1 (B, G, L, Q), Hes5-1 (C, H, M, R), Hes6-1 (D, I, N, S), and NeuroD (E, J, O, T) were examined. A, F, K, P show GFP expressing electroporated cells (arrowheads) and the corresponding area is indicated with arrowheads in the rest of the panels. Note that Notch1ICD apparently leads to decreased Hes1 expression (red arrowhead in L and arrowheads in Q) and induces Hes5-1 expression (M, R) and repress Hes6-1 (N, S) and NeuroD (O, T) expression regardless of the presence of Ngn3.
unknown whether NeuroD can induce Ngn3 expression, and the sensitivity of these bHLH factors to Notch mediated inhibition in endocrine development has not previously been investigated. To test if the Neurogenin-NeuroD cascade is unidirectional in endocrine development we compared the effect of introducing Ngn3 and NeuroD. We analyzed Ngn3 expression by in situ hybridization in NeuroD electroporated cells but found no evidence to suggest that NeuroD induce Ngn3 expression (not shown), consistent with similar studies in the nervous system [39] and indicating that NeuroD activates the differentiation program independent of Ngn3. However, we cannot exclude a transient induction of Ngn3 at earlier timepoints not analyzed. Next we examined if Notch1ICD could block the differentiation induced by Ngn3 or NeuroD by performing co-electroporation experiments (Fig. 7). In these experiments we targeted the prospective duodenum where no endocrine development occurs at the stages examined unless Ngn3 is induced and where delamination of differentiating endocrine cells is more readily scored [11]. We used an antibody to laminin to visualize the basal lamina of the endodermal epithelium and Pax6, glucagon, somatostatin and neuronal classIII β-tubulin [41] specific antibodies as endocrine markers. In our experiments both Ngn3 and NeuroD induced endocrine differentiation as evidenced by Pax6-, glucagon-, somatostatin-, and neuronal classIII β-tubulin expression and delamination of the differentiated cells (Fig. 7A,B and 7E,F). However, when Notch1ICD was co-expressed with either Ngn3 or NeuroD we found an almost complete loss of all these endocrine markers (Fig. 7C,D and 7G,H), showing that during forced endocrine differentiation both Ngn3 and NeuroD function is sensitive to Notch. Delamination of Ngn3 and NeuroD expressing cells still occurred under these conditions (Fig. 7, compare A to C and E to G), showing that delamination and expression of differentiation markers can be uncoupled during endocrine development.

**Myt1 can partially antagonize the effects of Notch1ICD on Ngn3 function**

The above experiments demonstrate that activated Notch1 can inhibit both Ngn3- and NeuroD-induced endocrine development. Since certain Ngn3-induced gene products, such as Hes6-1 and MyT1, have been reported to attenuate Notch mediated inhibition [42,43] we wanted to analyze if expression of any of these genes was insensitive to Notch inhibition in the duodenum, thus possibly explaining the delamination observed in the presence of activated Notch. As mentioned above (Fig. 6 and 7), both native and Ngn3-induced NeuroD and Hes6-1 expression in the pancreas is sensitive to Notch inhibition but delamination is not readily scored in the pancreas due to the branching of the epithelium. We therefore repeated the experiment in the duodenum and found that Ngn3-
induced expression of these two genes as well as Myt1 was repressed by Notch1ICD also at that location (Fig. 8A–H). This result suggests that Ngn3 can initiate a delamination program independent of the activity of these genes and that Ngn3 may have actions insensitive to Notch repression.

The levels of Notch1ICD resulting from forced expression are likely much higher than found during native Notch signaling events. Our experiments therefore do not rule out that Hes6-1 and/or Myt1 could render cells refractive to the action of more physiological levels of Notch as seen in neurogenesis [43]. To test if forced expression of Myt1 at high levels could antagonize Notch1ICD function we electroporated chicken endoderm with constructs encoding Ngn3, Notch1ICD and a FLAG tagged Nzf2b isoform of Myt1. This is one of two Myt1 isoforms resulting from alternative transcriptional start sites reported to be expressed at highest levels in the mouse pancreas. Furthermore, it is the only isoform capable of initiating the endocrine differentiation program in the chicken endoderm [44]. We found that expression of Myt1 led to a partial restoration of neuronal classIII β-tubulin expression (Fig. 8I–K), indicating that some aspects of endocrine differentiation have been rescued in these cells. Expression of Pax6, glucagon and somatostatin was, however, not restored under these conditions (not shown), suggesting that other factors are needed to overcome the repression by Notch1ICD.

Discussion

The pancreatic expression of bHLH genes and their sensitivity to Notch mediated inhibition observed in this study suggests that endocrine development is highly conserved between mice and chicken.

Overexpression of Notch ligands leads to endocrine differentiation

The observed stimulatory effect of Delta1 over-expression upon endocrine differentiation is in contrast to the effect of Delta1 over-expression in the chicken retina where widespread RCAS-mediated expression of Delta1 inhibits neuronal differentiation [45], and seemingly at odds with the proposed function of Delta1 as a signal that inhibits endocrine differentiation. However, high level expression of Notch ligands has previously been reported by a number of groups to result in cell autonomous inhibition of Notch signal reception [28-33], raising the possibility that the effect we observe is cell autonomous and the result of inhibition of Notch signaling rather than stimulation of Notch signaling in neighboring cells. In our experiments we used plasmid-based electroporation which results in multiple copies being taken up by the electroporated cells which combined with the very strong promoter used may result in expression levels exceeding those obtained by Henriques and colleagues who used an RCAS retrovirus-mediated delivery [45]. Furthermore, expression of a truncated signaling-defective version of Serrate-1 also resulted in enhanced endocrine development, arguing that the effects we observe with full-length Delta1 and Serrate-1 is the result of a dominant negative effect. This was further corroborated by the ability of Notch1ICD to reverse the effect of the ligands in a cell autonomous manner. However, in spite of the cell-autonomous effect on Notch signal reception, we would expect that cells lying adjacent to cells expressing Delta1 or Serrate-1 would be prevented from differentiating through the non-cell autonomous activation of Notch signaling in neighboring cells, while this effect should not be observed for cells lying next to Serrate-d1-expressing cells which are signaling defective. However, since differentiating endocrine cells delaminate from the epithelium we were not able to ascertain whether differentiation of cells initially lying adjacent to Delta1- or Serrate1-expressing cells was delayed or inhibited.

It is noteworthy that we always observe that a fraction of the ligand expressing cells fail to undergo endocrine dif-
ferentiation and that this fraction increases with time. One possible explanation for this is that these cells are differentiating into acinar cells as premature acinar differentiation is observed in zebrafish with compromised Notch signaling [19]; however, we found no evidence to suggest that amylose expression occurred prematurely. It is also possible that such cells are committed to a non-endocrine fate and that inhibition of Notch signaling therefore cannot induce endocrine differentiation. In this regard it is notable that forced expression of Ngn3 also fails to induce endocrine differentiation of all the cells that express the introduced gene. However, a trivial explanation may be that the cells that fail to differentiate express the introduced genes at levels that are incompatible with endocrine differentiation.

The endocrinogenic effect of Notch ligand expression was confined to the pancreatic endoderm. In spite of widespread expression in prospective duodenum we found no signs of endocrine development. This is most likely due to the restriction of Ngn3 expression to the pancreas anlage at this stage, but apparently contrasts with recent data from Gu and colleagues who found that forced expression of Manic Fringe in prospective duodenum led to endocrine development through inhibition of Notch signaling [46]. The reason for this discrepancy is unclear at present but could be related to the different mechanisms by which Manic Fringe and high levels of Delta1 inhibits Notch signal reception.

**Activated Notch inhibits endocrine promoting Ngn3 activity**

Previous studies of Notch function in pancreatic endocrine development have not considered a potential role for Notch mediated inhibition of Ngn3 and/or NeuroD protein function. Several examples of post-translational inhibition of pro-neural function by Notch have been described in the literature. The function of the exocrine bHLH factor p48-PTF1 has been shown to be inhibited via heterodimerization to Hes1 [19]. In Drosophila, several Notch target genes of the E(Spl) class have been shown to dimerize with pro-neural bHLH factors and antagonize the function of these [14], in Xenopus X-NGN1 function can be antagonized by activated Notch1 [39,40], and in human cell lines Notch signaling has been demonstrated to lead to a rapid degradation of the human achaete-scute homolog hASH1 [15]. It is possible that similar mechanisms regulate Ngn3 function. It is also possible that regulatory sequences of genes induced by Ngn3 also contain Hes binding sites and that Hes repressor activity is dominant over Ngn3. Although our results do not prove that Notch signaling is active in Ngn3-expressing precursors they do raise the possibility that Notch might have a second effect in endocrine differentiation in that it could regulate the levels and duration of Ngn3 activity. A similar but not identical experiment has been conducted by Murtough and colleagues who used Ngn3-Cre mice to conditionally mix-express Notch1ICD in endocrine progenitor cells which led to inhibition of endocrine differentiation [18]. However, since Ngn3 expression was not investigated in that study it is unclear whether this block in endocrine development was caused by inhibition of Ngn3 gene expression or Ngn3 protein activity. Recently, Notch1ICD was shown to re-direct endocrine progenitors to a duct cell fate when expression was induced in Pax4 expressing cells [47]. Presumably, this effect would require inhibition of Ngn3 as Pax4 is a target of Ngn3.

A surprising finding is that activated Notch did not result in significant upregulation of Hes1 in the pancreas but rather appeared to repress the expression of this Notch effector gene. As discussed above this may be a secondary effect to the induction of Hes5-1. In a transgenic mouse expressing Notch1ICD under the Pdx1 promoter, Hes1 protein could not be detected in most of the Notch1ICD expressing pancreatic cells even though Ngn3 expression was almost completely abolished throughout the epithelium[17]. It was speculated that the Hes1 protein is expressed at levels too low for detection by immunohistochemistry. An alternative interpretation is that the effect of Notch1ICD is mediated by other Hes factors such as Hes5, or that Notch signaling may be required but not sufficient for Hes1 expression under normal circumstances.

Surprisingly, Ngn3-induced delamination still occurred in the presence of Notch1ICD. The molecular mechanism controlling delamination of endocrine cells is poorly defined but it is possible that Ngn3-targets responsible for delamination are less sensitive to Notch-mediated repression than targets genes involved in endocrine differentiation, e.g., if genes required for delamination lack Hes binding sites in their regulatory elements. Intriguingly, differentiation and delamination is also uncoupled in other circumstances. A mutant Ngn3 protein carrying a missense mutation (R107S) is hypomorphic in regard to endocrine-inducing activity, but still capable of inducing delamination to the same extent as wild-type Ngn3 [48].

**Activated Notch antagonize Ngn3 as well as NeuroD function**

Conditional expression of Notch1ICD in mature insulin-producing beta cells has no apparent effect on the beta cell phenotype [18]. Since NeuroD function is required for beta cell survival and function [49,50] we speculated that NeuroD might be refractive to the effects of activated Notch. However, endocrine differentiation induced by forced NeuroD expression in chicken endoderm was completely prevented by co-expression of Notch1ICD. This could reflect a mechanistic change in NeuroD function in mature endocrine cells compared to precursor cells. In
agreement with this notion we found that a few endocrine cells in the pancreas expressed Notch1ICD probably reflecting the occasional transfection of existing endocrine cells at the time of electroporation. Alternatively, it is possible that the ROSA26 locus, used to conditionally express Notch1ICD by Murtaugh and colleagues, results in appreciably lower levels of expression than we obtain by electroporation and that high level expression of Notch1ICD is required for repression of NeuroD function. NeuroD is also capable of inducing neurogenesis when expressed in Xenopus ectoderm [39,51] but it is unclear if activated Notch can inhibit this function of NeuroD. In one study, X-NGNR-1 and XNeuroD were equally sensitive to inhibition by X-Notch1ICD in side-by-side comparisons [39], while in another study the number of neurons formed in response to X-NGNR-1 was reduced by X-Notch1ICD which was not the case for XNeuroD induced neurons [40]. A number of observations support a similar function in pancreatic endocrine development; forced expression of either Ngn3 or NeuroD in the pancreas can induce the endocrine differentiation program [8,10,11] and Ngn3 induces NeuroD expression [10,11], while NeuroD cannot induce Ngn3 expression (this study) suggesting that a unidirectional bHLH cascade may also regulate endocrine determination and differentiation.

**MyT1 antagonism of Notch1ICD activity**

During Xenopus neurogenesis MyT1 can in cooperation with X-NGNR-1 overcome the repressive activity of Notch upon X-NGNR-1 [43]. We observe only a partial antagonistic effect of MyT1 on Notch mediated repression of Ngn3 function. The reasons for this incomplete rescue is unclear but it is possible that the chicken electroporation system results in far higher levels of activated Notch than seen with mRNA injection in Xenopus oocytes. It is possible that MyT1 would have a greater effect if more physiological expression levels were used. Nevertheless, the expression of known Notch signaling modifiers such as Hes6-1, Myt1, and Manic Fringe in endocrine precursors [44,46] suggests that Notch signaling is finely regulated during the early stages of endocrine differentiation. However, conditional loss-of-function studies in mice are needed to further elucidate the cellular and biochemical mechanisms of Notch signaling during these early stages of endocrine development.

**Conclusion**

Together the data presented here establish that pancreatic endocrine development in the chicken relies in a conserved bHLH cascade under inhibitory control of Notch signaling. While this may not be surprising it does lay the ground for further studies that take advantage of the ease at which chicken embryos can be manipulated.

Our results also raise the possibility that Notch could act in Ngn3-expressing cells and delay their differentiation. To determine whether Notch in fact does act upon Ngn3 in vivo will require sophisticated conditional mutagenesis.

Lastly, our results demonstrate that expression of differentiation markers can be uncoupled from the process of delamination of differentiating cells from the epithelium.

**Methods**

**DNA constructs**

All expression constructs were made in a pCIG vector [52] optimized for EGFP intensity (pCIG5 or pCGIG5 with a Gateway (invitrogen) recombination cassette, kindly provided by Anne Grapin-Botton). A pcDNAI vector containing a cDNA encoding N-terminal haemaglutinin (HA) tagged mouse Ngn3 was kindly provided by François Guillemot. This vector was cut with HindIII and filled with klenow. The cDNA was released with NotI and the entire fragment was cloned into the EcoRV/NotI sites of pCIG5. The cDNA encoding N-terminally HA tagged rat NeuroD has been described previously [53]. The coding sequence was amplified by PCR with the primers CACCAT-GGGCTACCCATAGAT and CTAA TCGTGAAAGAT-GCCATT and TOPO cloned into the pENTR/D vector (Invitrogen). Subsequently, HA-NeuroD was cloned by recombination into pCGIG5. The cDNA encoding C-terminally myc tagged rat Notch1ICD has been described previously [17]. An EcoRI fragment containing the entire coding sequence was cloned into the EcoRI site of pCIG5. The mouse Myt1-7zf gene was FLAG tagged (MDYKD-DDDKMTKYSESGL) at the N terminus using PCR and cloned into pENTR/D (invitrogen). The tagged version of Myt1-7zf was cloned into the pCGIG5 vector using gateway cloning. The cDNAs encoding FLAG tagged chicken Delta1 and HA tagged chicken Serrate1 and the dominant negative Serrate1-d1 were a kind gift from Ken-ichi Katoh [28]. Delta1-FLAG was released with Apal (Klenow blunted) and BamHI and ligated to EcoRI (Klenow blunted) and BamHI sites in pEnt 2B (Invitrogen). The coding sequence was cloned into the pCGIG vector by Gateway recombination. The Serrate constructs were cloned into XhoI and EcoRI sites of pEnt 2B and subsequently inserted in the pCGIG vector by Gateway cloning. All constructs were verified by sequencing.

**In ovo endoderm electroporation**

Electroporations were performed as described in [11]. Briefly, fertilized White Leghorn eggs (Triova, Denmark) were incubated at 38°C, 50% relative humidity. Electroporation was performed on chicken embryos at HH st. 10-15 [23]: The eggs were windowed and a solution of DNA (with a fixed concentration of 2 µg/µl in PBS w/o Ca2+ and Mg2+, 1 mM MgCl2, 3 mg/ml carboxymethyl-
cellulose, 0.66 mg/ml Fast Green) was injected into the blastocoel. An anode was placed over and a cathode under the embryo along the anteroposterior axis of the embryo using a micromanipulator. Depending on stage, three to five square 50 ms pulses of 7–15 V were applied from a BTX ECM830 electroporator and the eggs were sealed and returned to the incubator and allowed to develop for 52–72 hours. Electroporated embryos were immunostained with an antibody specific for the epitope tags and co-localization with GFP was assayed: As expected we found that most GFP positive cells also expressed the epitope tags (not shown) and we therefore used GFP as a measure of productive expression. For co-expression experiments double immunohistochemistry against the different epitope tags was used to verify co-expression (not shown).

**Immunohistochemistry and statistical analyses**

Whole mount immunofluorescent stainings: The stomach with the proximal duodenum along with the pancreas was dissected from the embryos and fixed over night in 4% PFA at 4°C over night, equilibrated in 30% sucrose in PBS and frozen in Tissue-Tek OCT compound (Sakura finetek). 10 µm sections were obtained on a Leica CM3050 S cryostat and collected on SuperFrost plus (Menzel-GLäser) slides. Sections were washed in PBS and blocked for at least 1 hour in 0.5% TNB (PerkinElmer). Primary antibodies were diluted in 0.5% TNB and applied over night. Secondary antibodies were applied for 1 hour after several washes in PBS and slides were mounted in 20% glycerol (in 50 mM Tris buffer adjusted to pH8.4). Primary antibodies were: Mouse-anti-HA (HA.11, Babco), rabbit-anti-myc (A14, Santa Cruz), mouse-anti-FLAG (M2, Sigma), Mouse-anti-glucagon (Glu-001, Novo Nordisk A/S), guinea pig-anti-insulin (ab7842, Abcam), rabbit-anti-somatostatin (A566, DAKO), rabbit-anti-amylose (A 8273, Sigma), rabbit-anti-Laminin (19393, Sigma), mouse-anti-MPM2 (M3514, DAKO), rabbit-anti-βIII-Tubulin (PRB-435P, Nordic Biosite). rabbit-anti-Nkx6.1 [55], rabbit-anti-GFP (632460, Clontech), (DSHB) The mouse anti-Pax6 monoclonal antibody developed by Atsushi Kawakami was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Secondary antibodies were purchased from Jackson ImmunoResearch (Cy3 conjugated donkey-anti-rabbit and donkey-anti-guinea pig and Cy5 conjugated donkey-anti-mouse). Negative controls include omission of primary antibodies and staining of non-electroporated embryos Images were collected by confocal microscopy on an LSM 510 META Laser Scanning Microscope (Carl Zeiss) and fluorescent signals were assigned false colors with Zeiss LSM software. Quantification was performed by sampling sections covering all the electroporated endoderm (between 7 and 10 sections from each embryo) and counting at least between 350 and 750 GFP+ cells from embryos electroporated at HH st. 10–12 and between 1200 and 4800 for embryos electroporated at HH st. 13–15. Between 3 and 13 embryos were analyzed quantitatively for each condition. Statistical analyses were performed using Student’s T-test.

**In situ hybridization**

Digoxigenin labelled cRNA probes were generated by in vitro transcription using reagents from Roche according to manufacturer’s instruction. The following chicken cDNA clones were used for probe generation: Ngn3 was kindly provided by A. Grapin-Botton and J. M. Matter. Delta1, Notch1, Serrat1 and Serrate2 were generous gifts from D. Henrique [56,57]. NeuroD: ChEST695k11, Hes1: ChEST900h16, Hes6-1: ChEST29617, Hes6-2: ChEST529e6, Hes5-1: ChEST295e19, Myt1: ChEST672b12, Notch2: ChEST909j12. ChEST clones were from the BBSRC Chick EST database and purchased from MRC gene service. Wholemount ISH was performed essentially as in [26]. ISH on frozen sections: Frozen sections were thawed and denatured cRNA probes diluted to 1 ng/µl in hybridization solution (50% formamide, 1x salt, 10% Dextran sulphate, 1 mg/ml yeast tRNA, 1x Denhardt’s) were added directly to the sections. The sections were covered with a cover slip and placed in a humidified chamber (humidified with 5×SCC, 50% formamide) at 65°C over night. The slides were washed 3 times in wash solution (50% formamide, 1× SCC, 0.1% Tween-20) at 65°C followed by several washes in MABT (100 mM maleic acid, 150 mM NaCl, 0.1% Tween-20, adjusted to pH7.5 with NaOH) at room temperature. The sections were blocked in 2% blocking reagent (Roche), 20% heat inactivated goat serum in MABT and then equilibrated in NTMT (50 mM Tris, 0.1% Tween-20, pH7.5) at room temperature. The sections were covered with a cover slip and incubated over night with an AP conjugated sheep-anti-DIG (Roche) (1:2500 in blocking solution). The sections were washed in MABT and then equilibrated in NTMT (50 mM Tris, 0.1 M NaCl, 0.02 M MgCl₂, 0.1% Tween-20, pH9.5). AP activity was visualized with NBT/BCIP in NTMT. Images were collected with a Hamamatsu C5810 cooled CCD camera mounted on an Olympus BX51 microscope and processed in Adobe Photoshop™.

**Authors’ contributions**

JA-R designed and carried out most of the experiments. JH and AB performed the Notch ligand mis-expression. HY and JH-S cloned MyT1 and contributed to the mis-expression studies. JA-R and PS conceived the study, participated in its design and coordination, carried out the data analysis, and drafted the manuscript.

**Acknowledgements**

We are grateful to Louise C. Rosenberg for technical assistance and to Anne Grapin-Botton, Jean-Marc Matter, Domingos Henrique, Francois...
Guillemot, and Ken-ichi Katsube for reagents. P.S. was supported by JDRF and EU 6th Framework Program.

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