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Neurogenin 3-expressing progenitor cells in the gastrointestinal tract differentiate into both endocrine and non-endocrine cell types

Susan E. Schonhoff,[a,b] Maryann Giel-Moloney,[a,c] and Andrew B. Leiter[a,b,c,*]

[a] *Division of Gastroenterology, GRASP Digestive Disease Center, Tufts-New England Medical Center, Boston, MA 02111, USA*
[b] *Genetics Program, Tufts University School of Medicine, Boston, MA 02111, USA*
[c] *Department of Physiology, Tufts University School of Medicine, Boston, MA 02111, USA*

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Abstract

Mice deficient for the transcription factor *neurogenin 3* (*ngn3*) fail to develop endocrine cells in the intestine and pancreas and show partial endocrine differentiation in the stomach. We expressed Cre recombinase under control of a *ngn3* BAC to achieve high fidelity cell lineage tracing in vivo to determine whether endocrine cells in these organs differentiate from NGN3+ precursor cells. Our results indicate that all small intestinal enteroendocrine cells arise from *ngn3*-expressing cells and confirm that NGN3+ cells give rise to all pancreatic endocrine cells as noted previously. By examining mice at a developmental stage when all of the cell types in the stomach have differentiated, we have delineated region-associated differences in endocrine differentiation. A much smaller fraction of endocrine cells populating the acid-producing region of the stomach is derived from NGN3+ precursor in contrast to the antral–pyloric region. Unexpectedly, *ngn3* is expressed in cells that adopt non-endocrine cell fates including significant fractions of goblet and Paneth cells in the intestine and a small number of duct and acinar cells in the pancreas. Rarely, *ngn3* was expressed in pluripotent cells in intestinal crypts with resultant labeling of an entire crypt–villus unit. Thus, *ngn3* expression occurs in mixed populations of immature cells that are not irreversibly committed to endocrine differentiation.

Keywords: Neurogenin 3; Enteroendocrine; Goblet; Paneth; Gastrointestinal tract; Cell fate

Introduction

The gastrointestinal tract represents the largest endocrine organ in mammals, consisting of scattered individual cells in the stomach, small intestine, and colon, and as clusters of cells in the pancreatic islets of Langerhans. Unlike many endocrine glands, the cells of the gastrointestinal endocrine system differentiate in tissues where the overwhelming majority of surrounding cells are non-endocrine cells including acinar and ductal cells in the pancreas, enterocytes, goblet cells, and Paneth cells in the intestine, and parietal, mucous, and chief cells in stomach.

Gastrointestinal endocrine cells appear to differentiate from their neighboring non-endocrine cells by transmitting active Notch signals to adjacent cells to inhibit them from adopting an endocrine cell fate. Mice deficient for Hes1, a terminal effector of Notch signaling, are notable for precocious endocrine differentiation in the stomach and intestine, with three to seven times the normal number of different endocrine lineages (Jensen et al., 2000). In the pancreas, disruption of Notch signaling results in excessive early appearance of endocrine cells with depletion of progenitor cells and failure to develop the exocrine pancreas (Apelqvist et al., 1999). Conversely, activation of Notch within pancreatic progenitor cells prevents the differentiation of both endocrine and exocrine tissue, while activation within mature endocrine cells does not affect their differentiation state (Murtaugh et al., 2003). As is the case in the nervous system and other tissues, Notch signals appear to inhibit endocrine cell fate by repressing the expression of basic helix loop helix (bHLH) transcription factors.

Abbreviations: bHLH, basic helix loop helix; *ngn3*, *neurogenin 3*; Cre, Cre recombinase; β-gal, β-galactosidase; GI, gastrointestinal.

* Corresponding author. Division of Gastroenterology #218, GRASP Digestive Disease Center, Tufts-New England Medical Center, 750 Washington Street, Boston, MA 02111. Fax: +1-617-636-4207.
E-mail address: aleiter@tufts-nemc.org (A.B. Leiter).

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The atonal-related bHLH protein, Neurogenin 3 (NGN3), has been identified as an important pro-endocrine transcription factor required for endocrine cells to differentiate in the gastrointestinal tract (Gradwohl et al., 2000; Jenny et al., 2002; Lee et al., 2002). NGN3 increases expression of the bHLH protein, BETA2/NeuroD (Huang et al., 2000), which is important for terminal differentiation of several enteroendocrine cell types and for normal islet morphogenesis (Naya et al., 1997). Although *ngn3* is required for endocrine differentiation in the GI tract, it is not expressed in hormone-producing cells as it switches off before terminal differentiation (Apelqvist et al., 1999; Schwitzgebel et al., 2000). Consistent with a role in pancreatic endocrine cell fate determination, expression of NGN3 during early pancreatic development under control of the Pdx1 promoter in transgenic mice resulted in increased numbers of cells expressing islet hormones (Apelqvist et al., 1999; Schwitzgebel et al., 2000).

The phenotype of *ngn3* knockout mice is notable for the absence of endocrine cells in the pancreatic islets with normal differentiation of duct and acinar cells (Gradwohl et al., 2000). *Ngn3*-deficient mice also fail to develop any enteroendocrine cells in the small intestine (Jenny et al., 2002). The absence of endocrine cells in the small intestine and pancreas suggests that expression of *ngn3* is a critical event required for these cells to differentiate. In contrast to the intestine and pancreas, loss of *ngn3* expression did not prevent the differentiation of all endocrine cells in the glandular stomach where serotonin, enterochromaffin-like cells, and ghrelin-expressing cells were present (Jenny et al., 2002; Lee et al., 2002), indicating that these lineages are not dependent upon NGN3 as a pro-endocrine transcription factor.

Although both gain and loss of function studies in transgenic mice suggest that *ngn3* expression is essential for much of the normal gastrointestinal endocrine differentiation, they do not establish that the affected populations arise from a *ngn3*-expressing precursor. The absence of *ngn3* expression in hormone-producing cells does not directly link *ngn3* expression to endocrine precursors and cannot rule out the possibility that *ngn3*-expressing cells do not become endocrine cells, but instead influence surrounding cells to adopt an endocrine cell fate. Several earlier studies used expression of stable reporter genes in transgenic mice under control of the *ngn3* gene for lineage tracing and showed transgene expression in some but not all endocrine cells in the intestine and the stomach (Jenny et al., 2002; Lee et al., 2001). While these studies suggested that some NGN3+ cells become endocrine cells, it was unclear whether all gastric and intestinal endocrine cells arise from NGN3+ cells. A more recent study clearly showed that all islet cells are descendents of NGN3+ cells by site-specific recombination using the Cre–loxP system, but did not examine the intestine or stomach (Gu et al., 2002).

In the present study, we have used site-specific recombination to permanently mark cells that express *ngn3* during development irrespective of their subsequent cell fate after *ngn3* expression ceases. As anticipated, all endocrine cells of the small intestine arise from *ngn3*-expressing precursor cells as do pancreatic endocrine cells as described previously (Gu et al., 2002). In contrast, most endocrine cells in the body of the stomach appear to differentiate from progenitors that do not express *ngn3*. Surprisingly, a small fraction of non-endocrine cells in the pancreas and intestine also appear to be descendants of cells that expressed *ngn3*, indicating that expression of *ngn3* by itself does not ensure cells will adopt an endocrine cell fate.

Methods

BAC transgene construction by homologous recombination in Escherichia coli

We introduced sequences encoding *Cre recombinase* that included a nuclear localization signal and simian virus 40 polyadenylation sequences (a gift from K. Kaestner, University of Pennsylvania) into a suicide vector (derived from pKD4 (Datsenko and Wanner, 2000)) upstream of a *frt* flanked *aminoglycoside phosphotransferase* gene (kanamycin resistant, *KANA*) to make pKD4-*nlsCreKANA*. PKD4-*nlsCreKANA* was used as a PCR template to amplify a fragment containing *nlsCreKANA* and 5′ and 3′ ends homologous to sequences flanking the mouse *neurogenin 3* ATG (PCR primers: sense = GCTGGCACACACACCTTCCAT TTTTTCCCAACCGCAGGATG-CCCAAGAAGAAGAGGAA, antisense = ACACTTGGATGGTGAGCGCATCCAAGGGAT-GAGGCGCCATCCTCCTTAGTTCCTA-TTCCGA, the underlined region is homologous to the sequence-flanking the *ngn3* translation start site). BAC clone # RPCI-23-121F10 was obtained from the Roswell Park BAC library (ResGen Invitrogen). Bacteria containing RPCI-23-121F10 BAC and expressing the λ phage red recombination system (Cotta-De-Almeida et al., 2003; Datsenko and Wanner, 2000) were made competent and transformed with the amplified fragment. Clones in which the amplified fragment was inserted via homologous recombination were selected using resistance to chloramphenicol and kanamycin. Selected colonies were screened for correct recombination by amplification using *Cre*- and *ngn3*-specific primers. The FRT-flanked *KANA* gene was excised from *ngn3-nlsCreKANA* BAC clones by expressing the FLP recombinase (Cotta-De-Almeida et al., 2003; Datsenko and Wanner, 2000). Southern blots, Field Inversion Gel Electrophoresis, and DNA sequencing confirmed correct transgene construction and integrity of the BAC flanking sequence.

Production of transgenic mice

The *ngn3-nls-Cre* BAC was linearized with AscI and purified for pronuclear microinjection (Hogan et al., 1994). Potential founder mice were genotyped by tail DNA ampli-

fication using primers specific for the *Cre* coding sequence. Transgenic pedigrees were maintained on a CD1 background. *Ngn3-Cre* transgenic mice were crossed to homozygous *R26R* mice (B6.129S4-Gt 26Sor^{tmSor}) from Jackson Laboratories (Soriano, 1999) to produce *ngn3-Cre X R26R* heterozygous mice.

Histology and immunohistochemistry

Tissue samples were fixed as described previously (Ratineau et al., 2000; Rindi et al., 1999) and processed for either paraffin or frozen sections. For Xgal histochemistry, tissues were fixed in 1.0% glutaraldehyde, 5 mM EGTA, in rinse buffer (PBS + 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.1% Triton X), and stained with rinse buffer containing Xgal solution (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 1 mg/ml Xgal) for 12–24 h and processed as described previously (Ratineau et al., 2000).

Slides were incubated with the following antibodies: rabbit anti-β-gal at 1:2500 (Cappel), rabbit anti-ghrelin at 1:2500 (#13–28, a gift from K. Kangawa, Osaka, Japan (Kojima et al., 1999)), rabbit anti-chromogranin A at 1:1000 (ImmunoStar), mouse anti-gastrin at 1:4000 (#E5, Cure, UCLA), rabbit anti-Cre at 1:10,000 (Novagen), rabbit anti-NGN3 at 1:1000 (a gift from M. German, UCSF), and rabbit anti-ki67 at 1:3000 (a gift from J. Gerdes, Borstel, Germany). Antisera against somatostatin and serotonin have been described previously (Lopez et al., 1995). Antibodies were detected with Cy3- or Cy2-conjugated secondary antibodies (Jackson Immunoresearch), the mouse on mouse immunodetection kit (Vector), or by tyramide signal amplification (TSA) (Molecular probes). Co-localization studies with two rabbit primaries was performed using a monovalent FAB fragment strategy (Negoescu et al., 1994) or by tyramide amplification (Brouns et al., 2002). In all cases, controls showed that the anti-rabbit IgG antibodies were unable to detect the first rabbit primary.

Lysozyme and *Wisteria floribunda* (WFA) lectin were visualized on Xgal-stained slides by standard immunoperoxidase detection (Ratineau et al., 2000) using rabbit anti-lysozyme at 1:500 (Zymed) with additional antigen retrieval (microwaved in 1 M urea) and using biotinylated-WFA at 1:600 (Sigma) with additional blocking of endogenous biotin (Vector). Schiff's periodic acid staining kit (Polyscience) was used for PAS staining.

Morphometric analysis

Multiple sections from the pancreas, stomach, small intestine, and colon from at least three mice were analyzed to determine the frequency of β-galactosidase expression in different cell types. In the pancreas, the percentage of acinar cells expressing β-galactosidase was estimated by dividing the area of Xgal-stained cells by the total acinar cell area (n = 4; total area, 582,244 μm^2; Xgal-stained area = 4333 μm^2). The total area examined per section ranged from 34,067 to 432,604 μm^2. The frequency of ducts containing cells arising from NGN3+ cells was determined by dividing the number of *W. floribunda* (WFA)-stained ducts containing Xgal+ cells by the total number of WFA-stained ducts from pancreatic sections (total ducts = 98; marked ducts = 14). A crude estimate of the frequency of duct cells arising from Ngn3+ cells was determined by dividing the number of Xgal+ duct cells by the total number of duct cells. Periodic longitudinal sections of the intestine and stomach at least 1 cm in length were examined from each animal. The percentage of goblet cells arising from NGN3+ cells was estimated by dividing the number of PAS+/Xgal+ cells by the total number of PAS-stained cells (duodenum: 531 PAS+ cells, 465 Xgal+ cells, 72 Xgal+/PAS+ cells; colon: 2820 PAS+, 259 Xgal+, 44Xgal+/PAS+). The percentage of Paneth cells arising from NGN3+ cells was estimated by dividing the number cells double-stained for lysozyme and β-galactosidase by the total number of lysozyme-stained cells (duodenum: 190 Lys+ cells, 18 Xgal+/Lys+; jejunum: 641 Lys+, 110 Xgal+/Lys+; ileum: 343 Lys+, 23 Xgal+/Lys+). The percentage of different endocrine cell types in the stomach arising from NGN3+ cells was determined by counting the number of cells stained for each hormone that showed staining for β-galactosidase. At minimum of 300 and 600, β-galactosidase-stained cells were examined in the body and antrum of the stomach, respectively, for each animal.

Results

Generation of transgenic mice expressing Cre recombinase under control of the neurogenin 3 gene

Cre encoding sequences containing a nuclear localization signal were inserted into exon 1 of the murine *ngn3* gene at the initiator methionine. To ensure that *Cre* expression would recapitulate normal cell-type specific and developmental expression of *ngn3*, we constructed the transgene in a BAC clone containing approximately 81 kb of the 5′ flanking sequence and 102 kb of the 3′ flanking sequence (Fig. 1A), introducing *Cre* by homologous recombination in *E. coli* using the phage λ red recombination system (Cotta-De-Almeida et al., 2003; Datsenko and Wanner, 2000; Yu et al., 2000). The homologous recombination system allowed us to precisely insert the *Cre* encoding sequences into the *ngn3* locus at the initiating methionine without deleting any endogenous *ngn3* sequences. The presence of SV40 *polyA* sequences at the end of the Cre transcript made it highly unlikely that expression of ngn3 from BAC sequences could arise from transcriptional read through. Two independent pedigrees of transgenic mice showed identical patterns of *Cre* expression and identical results in lineage tracing experiments.

In the small intestine, we identified scattered single cells showing immunostaining for Cre in a similar distribution to NGN3 at the base of the intestinal crypt (Fig. 1B). Like

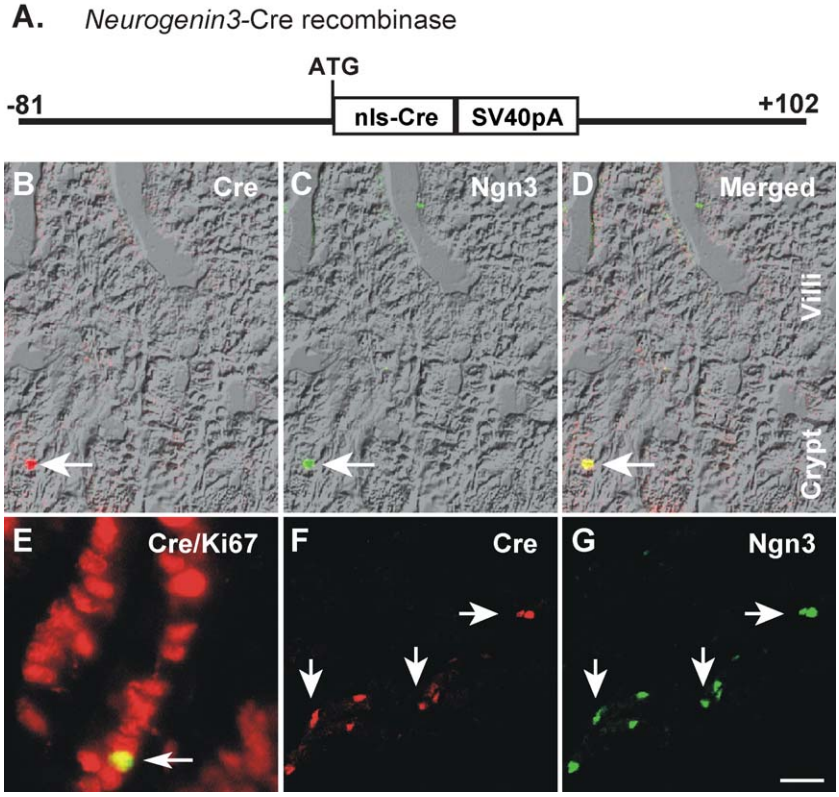

Fig. 1. *Cre* expression is directed to *ngn3*-expressing cells in transgenic mice. (A) Structure of the *ngn3-Cre* transgene. *Cre* was introduced into a BAC containing −81 to +102 of flanking sequence by homologous recombination in *E. coli.* (B–D) Co-expression of Cre and NGN3 in a single crypt cell nuclei in the small intestine of an adult transgenic mouse. Immunofluorescent staining shown for Cre (B, detected with Cy3, red) and NGN3 (C, detected with Alexafluor488, green), with the merged image (D) showing yellow double-stained cells. (E) Merged immunofluorescent image showing co-expression of Cre (green) and Ki67 (red) in small intestinal crypts. The Cre-positive nucleus (arrow) is Ki67+ and appears yellow. (F and G) Co-expression of Cre (F, red) and NGN3 (G, green) in epithelial cells at e16.5 in the pancreas. Scale bar = 80 (B–D), 12.5 (E), and 50 μm (F–G).

NGN3, Cre staining was restricted to the crypt and was never seen in villi. Double immunofluorescent staining for Cre and NGN3 showed that all cells staining for Cre also stained for NGN3, indicating that the BAC sequences directed *Cre* expression only to cells expressing the endogenous *ngn3* gene (Figs. 1B–D). *Cre* was expressed in dividing cells expressing Ki67, a marker of proliferating cells, indicating that the transgene was transiently expressed only in relatively immature cells in the small intestinal crypts similarly to NGN3 (Fig. 1E). In the fetal pancreas at embryonic day 16.5, we observed Cre single cells and clusters of cells throughout the tissue that co-localized with NGN3 (Figs. 1F and G).

Cell fate of neurogenin 3-expressing progenitor cells in the pancreas

Both *ngn3-Cre* transgenic lines were crossed with the modified reporter line, Rosa26 (*R26R*) (Soriano, 1999). The *ROSA26* locus directs generalized constitutive expression in most tissues, including the GI tract throughout development. The indicator strain was created by inserting *lacZ* into the *ROSA26* locus and modified by introducing floxed *polyA* stop sequences upstream of *lacZ*, thus preventing *lacZ* expression in the absence of Cre recombinase. Transcription read through and expression of *lacZ* are permanently activated by Cre-mediated recombination between the loxP sites to excise the stop sequences. As a result, even cells and their descendants that transiently express *ngn3* will be permanently marked by β-galactosidase expression independent of their eventual cell fate.

As expected, entire islets in the adult pancreas stained strongly for β-galactosidase activity in *ngn3-Cre X R26R* mice (Fig. 2A). All cells that stained for the secretory granule marker, chromogranin A, showed β-galactosidase immunostaining, confirming that all pancreatic endocrine cells arose from *ngn3*-expressing cells (Figs. 2B–D).

Unexpectedly, we identified small numbers of acinar and duct cells in the adult pancreas that expressed β-galactosidase using two independent pedigrees of *ngn3-Cre* transgenic mice. We never observed Cre or NGN3 expression in either non-endocrine lineage, indicating that β-galactosidase activation occurred as a result of transient *Cre* expression earlier in development. Small numbers of acinar cells (mean = 0.8%; range = 0.5–1.8%) showed staining for β-galactosidase activity (Fig. 2E). The β-galactosidase-expressing acinar cells always appeared as clusters, rather than single cells, appearing to stain an entire acinar branch. This suggests that small numbers of isolated epithelial progenitor cells express

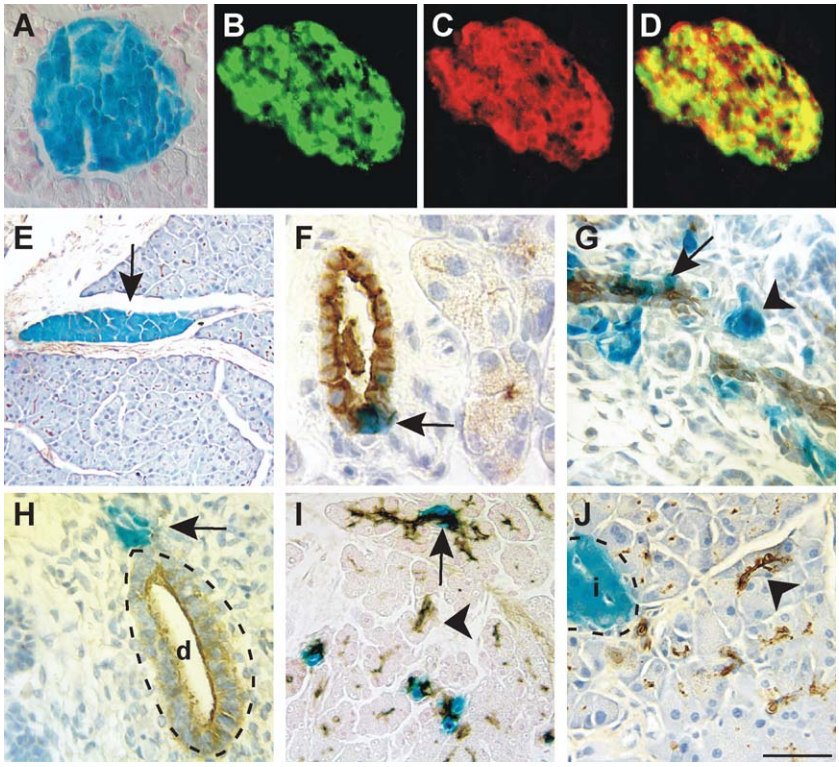

Fig. 2. Identification of descendants of NGN3+ cells in the pancreas of *ngn3-Cre* X *ROSA26* mice. (A) β-gal activity in an adult pancreatic islet detected by Xgal histochemistry. (B–D) Double immunofluorescent staining showing co-expression of chromogranin A (B, detected with Cy2, green) and β-gal (C, detected with Cy3, red); the merged image (D) shows all islet cells stain yellow. (E) β-gal activity in a cluster of acini cells (arrow) in adult pancreas. (F–J) Co-staining of β-gal activity and WFA (brown). (F) β-gal activity marks a few cells (arrow) within an adult WFA+ pancreatic duct. (G) At e15.5, both Xgal+/WFA− cells (arrowhead) and Xgal+/WFA+ cells are observed (arrow). (H) Fetal pancreas containing a duct with cuboidal epithelium (dashed line marked as "d"). Xgal+/WFA− cells are observed adjacent to the duct (arrow). (I) At e17.5, Xgal+/WFA+ cells are observed (arrow) along with Xgal−/WFA+ tubules (arrowhead). (J) In the adult pancreas, an Xgal+ islet is observed (dashed line marked as "i") along with WFA+ tubules not stained by Xgal (arrowhead). Scale bar = 39 (A), 42 (B–D), 122 (E), 40 (F–G), 37 (H), 70 (I), and 50 μm (J).

ngn3 and give rise to acinar cell clusters, which represent their clonal descendants. A significant fraction (14 ± 5%) of pancreatic ducts in *ngn3-Cre X R26R* mice contained one or more cells expressing β-galactosidase. Most ducts showed no staining with some ducts containing one to several stained cells. β-Galactosidase expression was most frequently observed in subsets of interlobular ducts and the major pancreatic ducts. We confirmed the identification of Xgal-stained cells as duct cells by showing co-staining for *W. floribunda* agglutinin (WFA), a lectin expressed on duct cells in adult mice (Lammert et al., 2001) (Fig. 2F). Despite the difficulty in accurately counting the total numbers of cells in the larger ducts, we estimated that between 1% and 5% of duct cells showed staining for Xgal, indicating that they arose from NGN3+ cells.

One question not addressed in the adult pancreas due to the relatively small fraction of β-galactosidase-labeled duct cells is whether endocrine cells arise from duct cells. Because duct-associated endocrine progenitors occur much more frequently in the fetal pancreas, we analyzed the pancreas of *ngn3-Cre X R26R* mice during fetal development. To determine the relationship between ducts and cells expressing β-galactosidase, we co-stained cells with *W. floribunda* agglutinin (Figs. 2G–J). A significant fraction of Xgal-stained cells did not stain for WFA at e15.5 (Fig. 2G, arrowhead). Large circular ductal structures with a cuboidal epithelium showed apical WFA staining. Most of these ducts did not stain for β-galactosidase (Fig. 2H), but an occasional duct contained one or two Xgal-stained cells, much like the ducts of the adult pancreas. We identified a distinct population of cells with surface staining for WFA that appeared to be branched tubular structures without the typical morphology of larger ducts. A fraction of these WFA-stained cells stained for β-galactosidase activity at e15.5 and e17.5 (Figs. 2G and I, arrows). In contrast, in the adult pancreas, tubular, acinar-associated WFA-positive cells did not stain for β-galactosidase (Fig. 2J, arrowhead). Thus, islet precursors appear to arise from a population of cells that express WFA but not from cuboidal cells in circular ducts.

Small intestinal enteroendocrine cells arise from ngn3-expressing cells

In the adult small intestine, *ngn3* expression is restricted to a relatively immature cell population deep in the crypts

of Lieberkuhn in close anatomic proximity to stem cells, suggesting that *ngn3* is expressed early during differentiation along the crypt–villus axis (Fig. 1C). As cells migrate up the crypt–villus axis, they stop dividing and terminally differentiate into one of three cell types: enteroendocrine cells, enterocytes, and goblet cells. Paneth cells, the fourth epithelial cell type, migrate down to the crypt base as they differentiate. We stained sections of the small intestine for both NGN3 and chromogranin A, a marker of secretory granules to determine if *ngn3* expression precedes endocrine differentiation. NGN3 and Cre were not expressed in chromogranin A-stained cells, indicating that *ngn3* expression precedes the appearance of markers of endocrine differentiation in the intestine (Fig. 3A). We analyzed the descendants of *ngn3*-expressing cells by staining for β-galactosidase in *ngn3-Cre X R26R* mice. In most cases, we observed scattered, individual cells expressing β-galactosidase distributed from the crypt to the villus tip in all regions of the intestine including the duodenum, jejunum, ileum, and colon (Figs. 3B and C). Because NGN3 expression is restricted to chromogranin A-negative cells located in the lower crypt, the β-galactosidase-stained cells represent descendants of immature nondifferentiated *ngn3*-expressing cells. To determine whether all enteroendocrine cells in the intestine arise from ngn3-expressing progenitors, we stained intestinal tissue for both chromogranin A and β-galactosidase (Figs. 3D and E). All chromogranin A-expressing cells showed staining for β-galactosidase, indicating that all intestinal endocrine cells expressed *ngn3* during some stage of differentiation.

Descendants of some ngn3 expressing cells adopt a non-endocrine cell fate in the intestine

Further examination of intestinal sections stained for both chromogranin A and β-galactosidase revealed small numbers of individual cells that stained with β-galactosidase but did not express the endocrine differentiation marker. Some of

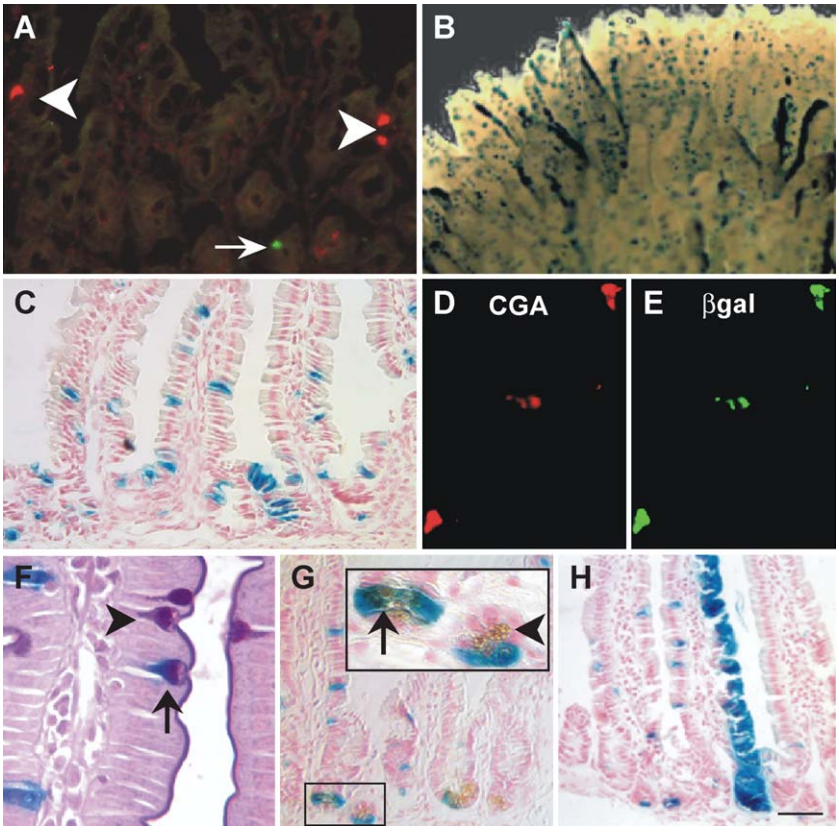

Fig. 3. Cell fate NGN3+ cells in the small intestine of *ngn3-Cre* X *ROSA26* mice. (A) Double immunofluorescent staining showing that Cre (detected with Alexafluor488, green) stained cells found at the base of the crypt (arrow) are immature and do not express chromogranin A (arrowheads, detected with Cy3, red). (B) A low power view of intestinal epithelium stained for β-gal activity detected by Xgal histochemistry (blue cytoplasmic staining). Scattered blue cells and rare villus strips show staining. (C) Longitudinal sections of intestinal epithelium stained for β-gal activity. Xgal cells are found scattered throughout the epithelium from the crypts to the tips of the villi. (D–E) Double immunofluorescent staining showing co-expression of chromogranin A (D, detected with Cy2 green) and β-gal (E, detected with Cy3, red). (F) Double staining for β-gal activity and mucins, detected with Periodic Acid Schiff's reagent, which appears dark pink/purple. PAS+/Xgal+ goblet cells (arrow) were observed as were PAS+/Xgal− goblet cells (arrowhead). (G) Paneth cells shown by immunoperoxidase staining for lysozyme (brown) and β-gal. A fraction of Paneth cells are Xgal+ (arrow) and others are Xgal− (arrowhead). (H) Longitudinal sections of the intestinal epithelium stained for β-gal activity showing Xgal marking an entire half-villus unit. Scale bar = 32 (A), 39 (C), 24 (D–E), 17 (F), 39 (G), and 50 μm (H).

these cells had the morphological appearance of either goblet cells or Paneth cells. We confirmed that some goblet cells arose from *ngn3*-expressing precursors by staining for mucins with PAS and for β-galactosidase activity with Xgal. We identified goblet cells within the same villus unit that were Xgal+, along with others that were not, suggesting that they arose from different precursor cells in the same crypt (Fig. 3F). Approximately 14% of goblet cells in the duodenum stained for β-galactosidase with the percentage declining as one moves caudally to as low as 1.6% to the colon. We identified a subset of Xgal+ Paneth cells (approximately 13%) that appeared to arise from NGN3+ cells (Fig. 3G). Labeling of goblet or Paneth cells occurred in both *ngn3-Cre* lines, and we never observed Cre expression in goblet or Paneth cells, suggesting that β-galactosidase activation occurred in their immature precursors that transiently expressed *Cre* rather than as a result of ectopic expression in these two lineages.

The fourth major epithelial cell type of the intestine, enterocytes, did not stain for β-galactosidase activity in most crypt–villus units. Instead, we occasionally observed a striking pattern of β-galactosidase staining in all cells on one side of a villus in longitudinal sections but not in individual enterocytes (Figs. 3B and H). The observed pattern of staining suggests that all epithelial cells that differentiate from a single crypt arose from a *ngn3*-expressing progenitor. This pattern of staining further suggests that some *ngn3*-expressing cells are pluripotent, possibly either a crypt stem cell or one of its immediate descendants, because all cells in such units are expressing β-galactosidase and continue to express the enzyme as the epithelium perpetually renews itself every 4–5 days.

Distinct regions of the stomach show predominantly ngn3-dependent or ngn3-independent endocrine differentiation

Little is known about the relationship between *ngn3*-expressing cells and endocrine cells populating the adult mouse stomach. *Ngn3*-expressing cells were seen extremely rarely in the stomach of adult mice where they co-expressed Cre (not shown). To determine the location and abundance of cells arising from *ngn3*-expressing precursors, we examined the antral- and acid-producing regions for β-galactosidase expression in *ngn3-Cre X R26R* mice. We identified numerous β-galactosidase-expressing cells at the base of the antral epithelium (Fig. 4A). β-Galactosidase-stained cells were scattered throughout the oxyntic epithelium at much lower abundance than the antrum (Fig. 4E).

Unlike the pancreas and intestine, many endocrine cells in the stomach of adult mice did not stain for β-galactosidase, indicating that they did not arise from cells that expressed *ngn3*. The majority, but not all, chromogranin A-expressing endocrine cells in the antrum also expressed β-galactosidase activity (81 ± 10%) in *ngn3-Cre X R26R* mice (Fig. 4B). Further characterization of antral endocrine cells revealed that most of these were gastrin-expressing cells (Fig. 4C). The majority of G-expressing (90 ± 3%), D-expressing (90 ± 8%), and ghrelin-expressing (82 ± 3%) cells expressed β-galactosidase (Fig. 4D and data not shown). Thus, most antral endocrine cells in adult mice are descendants of a *ngn3*-expressing progenitor.

In contrast to the antrum, a much smaller fraction (39 ± 3%) of chromogranin A-stained cells expressed β-galactosidase in the oxyntic epithelium, indicating that many endo-

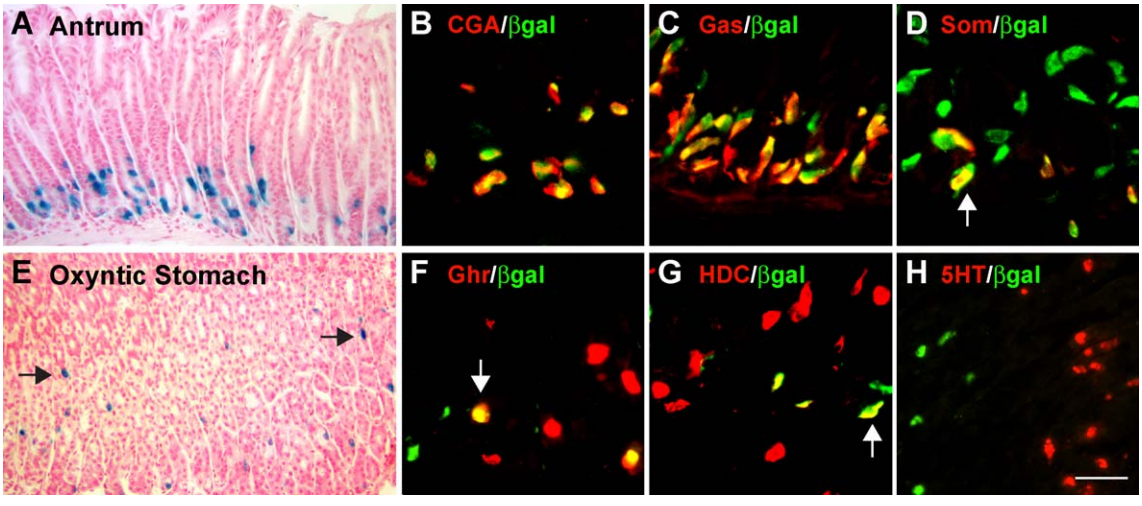

Fig. 4. Relationship between *ngn3*-expressing cells and endocrine cells in the adult mouse stomach. (A) β-Gal activity in the antrum of adult *ngn3-Cre X R26R* mice. Xgal+ cells are found at the base of the epithelium in the antrum. (B–D) Merged image of double immunofluorescent staining of the antral–pyloric region for β-gal (green) and chromogranin A, red (B); gastrin, red (C); and somatostatin, red (D). (E) β-Gal activity in the stomach body of adult *ngn3-Cre X R26R* mice. A few Xgal+ cells are scattered throughout the oxyntic region of the stomach (examples marked by black arrows). (F–H) Merged image of double immunofluorescent staining of the oxyntic region for β-gal (green) and ghrelin, red (F); histidine decarboxylase (HDC), red (G), or serotonin, red (H). Double-positive cells appear yellow, with examples marked by arrows. Scale bar (in panel H) = 34 (A), 29 (B), 28 (C), 17 (D), 96 (E), 38 (F), 40 (G), and 50 μm (H).

crine cells in the acid-producing region of the stomach of adult mice are not direct descendants of *ngn3*-expressing cells. Examination of major endocrine cell populations in the oxyntic region revealed β-galactosidase activity in similar fractions of ghrelin-expressing cells (41 ± 3%) and histidine decarboxylase-expressing (HDC) cells (35 ± 6%) (Figs. 4F and G), whereas none of the cells expressing serotonin stained for β-galactosidase (Fig. 4H), suggesting that serotonin-expressing cells do not arise from cells that expressed *ngn3*.

Discussion

The phenotype of *ngn3* knockout mice suggests that *neurogenin 3* plays an important role in the initiation of endocrine differentiation in the pancreas, small intestine, and a subset of cells in the stomach (Gradwohl et al., 2000; Jenny et al., 2002; Lee et al., 2002). However, studies with *ngn3* null mice have not established whether *ngn3*-expressing cells are endocrine precursor cells that directly give rise to mature endocrine cells. The absence of *ngn3* expression in cells expressing markers of endocrine differentiation has further limited identification of NGN3+ cells as endocrine precursor, presumably due to *ngn3* expression switching off before terminal differentiation (Apelqvist et al., 1999; Schwitzgebel et al., 2000). Several studies using transgenic mouse models have attempted to address whether NGN3+ cells are endocrine progenitor cells in the pancreas, intestine, and stomach. Site-specific recombination using the Cre–loxP system clearly marked all islet cells as descendants of NGN3+ cells (Gu et al., 2002). Additional studies directing the expression of stable reporter genes in transgenic mice under control of the *neurogenin 3* gene indicate that some endocrine cells in the intestine and stomach may arise from NGN3+ precursors (Jenny et al., 2002; Lee et al., 2001, 2002).

In the present work, we show several new and distinct findings that further elucidate the role of *ngn3* in gastrointestinal endocrine differentiation, revealing previously unappreciated region-specific differences within the gastric epithelium. First, using the Cre–LoxP system, we establish that all small intestinal enteroendocrine cells arise from NGN3+ progenitors. Examination of the cell fate of NGN3+ cells in the adult stomach revealed that most endocrine differentiation in the oxyntic region of the stomach does not occur through NGN3+ progenitor cells in contrast to the pancreas, small intestine, and the antral–pyloric region of the stomach. Lastly, we show that some non-endocrine cells in both the small intestine and pancreas are derived from cells that expressed *ngn3* at some stage of development, indicating that expression of *ngn3* by itself does not commit cells to adopt an endocrine cell fate. In addition, our work confirms earlier work that pancreatic endocrine cells arise from NGN3+ cells (Gu et al., 2002).

We have identified cells in the adult pancreas, small intestine, and stomach that arise from *ngn3*-expressing precursor cells by cell lineage tracing in *ngn3-Cre* × *ROSA26* transgenic mice (Soriano, 1999). In contrast to knockout models, this system allowed us to follow the cell fate of NGN3+ cells and to address whether endocrine cells in adult mice arise from NGN3+ cells. The *Cre* transgene contained 183 kb of the *ngn3* gene sequence to ensure inclusion of regulatory domains needed to mimic the temporal and spatial expression of *ngn3*. Two independent pedigrees gave identical results, further suggesting that enough sequence was present in the transgene to avoid integration site effects that could influence expression.

NGN3+ cells may differentiate into acinar and duct cells in the pancreas in addition to islets

An earlier study used 6.5 kb of the 5′ flanking sequence of the *ngn3* gene to direct expression of Cre recombinase. In this study, *ngn3-Cre* mice were crossed to the Z/AP reporter mouse to mark NGN3+ cells and to trace their subsequent cell fate in the pancreas. All cells within the islets were identified as descendants of NGN3+ cells with minimal labeling of duct (<0.1%) or acinar (<0.1%) cells (Gu et al., 2002). By expressing Cre under control of a much larger genomic fragment, we found that acinar and duct cells originated from *ngn3*-expressing cells at a much higher frequency and identified clusters of acinar cells rather than single cells, suggesting they arose from expansion of infrequently occurring early NGN3+ acinar cell precursors. These marked duct and acinar cells may have originated from a subset of relatively undifferentiated precursor cells that expressed *ngn3* and *Cre* at low levels below a threshold needed for endocrine cell specification, but above that required for efficient recombination. The failure to mark these non-endocrine populations in the earlier study may have resulted from the absence of regulatory elements in the 6.5 kb transgene required for expression in these immature cells or from increased susceptibility of short transgenes to positional effects of transgene integration. The use of longer BAC transgenes greatly reduces but does not entirely eliminate the possibility of obtaining misleading information due to abnormal chromosomal integration. The nearly identical findings in two independent pedigrees strongly suggest that transgene expression was not subject to integration position effects.

Before the formation of discrete islets in the fetal pancreas, hormone-expressing cells are frequently associated with duct-like structures (Herrera et al., 1991; Pictet et al., 1972). These findings have raised the possibility that islets may differentiate from progenitor cells in pancreatic ducts. Our work, as also shown by Gu et al. (2002), shows that most mature ducts do not contain cells that once expressed *ngn3*. However, we noted occasional *ngn3* marked adult pancreatic duct cells. In most cases, a single duct contained only one or two marked cells, suggesting that *ngn3* was expressed relatively late in a duct cell, rather than in an early expanding progenitor. Another possible interpretation of the labeled

cells in adult ducts is that a progenitor in fetal ducts expressed *ngn3* and gave rise to a clone of cells, most of which moved out of the duct to form islet endocrine cells in the adult, leaving behind a few marked duct cells.

If this were true, one prediction is that many more duct cells in the fetal pancreas should be labeled before birth. We observed no difference between fetal and adult mice in the frequency of *ngn3*-expressing descendants in medium-sized circular ducts lined with cuboidal epithelium. Many tubular duct-like structures in the fetal pancreas, which stained for a lectin (WFA) seen in adult ducts, appear to have expressed *ngn3*. It is unclear whether these cells represent true duct cells or duct cell progenitors. Until more specific markers for duct cells and their progenitors become available, it will be difficult to establish the relative contribution of pancreatic ducts, if any, to islets.

Endocrine differentiation in the oxyntic stomach is mostly ngn3-independent

Analysis of *ngn3* null mice provided the first indication that endocrine differentiation in the stomach was not entirely dependent on *ngn3* expression (Jenny et al., 2002; Lee et al., 2002). Whereas gastrin-, somatostatin-, and glucagon-expressing cells failed to develop in the absence of *ngn3*, significant numbers of cells expressing ghrelin and serotonin were identified. Ngn3−/− mice die before the appearance of histamine-containing enterochromaffin-like cells (ECL). However, isografts from *ngn3*−/− stomachs transplanted into recipient animals developed ECL cells, suggesting that differentiation of this population was not controlled by *ngn3* (Jenny et al., 2002). However, it is difficult to estimate the relative contribution of NGN3+ cells to all stomach endocrine cells because *ngn3*-null mice fail to survive more than 3 days after birth, before maturation of the gastric mucosa (Gradwohl et al., 2000).

Enhanced green fluorescent protein knocked into the *ngn3* locus appeared to be expressed in some gastric endocrine cells, indicating that they arose from NGN3+ cells (Lee et al., 2002). However, it is difficult to assess the relative contribution of NGN3+ cells to mature endocrine cells because many endocrine cells were not labeled, possibly as a result of transient EGFP expression before differentiation. Jenny et al. (2002), using a *lacZ* reporter gene directed by 6.9 kb of *neurogenin 3* flanking sequences, showed β-galactosidase was co-expressed with endocrine cells expressing gastrin, somatostatin, ghrelin, and serotonin in newborn mice, and in ECL cells of adult mice (Jenny et al., 2002).

In contrast to these earlier studies (Jenny et al., 2002; Lee et al., 2002), we carried out our cell lineage analysis in adult mice at a stage when the gastric epithelium is fully developed. At this stage, the stomach is comprised of anatomically, histologically, and functionally distinct regions, the oxyntic region, which produces gastric acid, and the antrum, which contains a large population of endocrine cells involved in the regulation of acid secretion. Analysis of these two distinct regions revealed striking differences in the relationship between endocrine cells and NGN3+ cells not appreciated from earlier work studying newborn animals.

The majority of, but not all, endocrine cells in the antral stomach of adult mice appear to arise from *ngn3*-expressing precursors, especially gastrin-expressing G cells and somatostatin-expressing D cells. However, in *ngn3* null mice, expression of somatostatin was entirely eliminated. Our observations may indicate that in adult mice, differentiation of D cells becomes partially *ngn3*-independent in contrast to neonatal animals.

Endocrine differentiation in the oxyntic portion of the stomach appears to differ significantly from the antral–pyloric region. Less than 40% of chromogranin A-, ghrelin-, and enterochromaffin-like cells in the oxyntic stomach and none of the serotonin-expressing cells arise from NGN3+ precursors. Our observations appear to differ somewhat from lineage analysis using *ngn3-lacZ* mice where transgene expression was identified in all endocrine cell types. The apparent differences may be related to the age of the animals studied or possibly due to misexpression of the relatively short transgene (Jenny et al., 2002). At this time, the factors that control *ngn3*-independent endocrine cell fate specification in the stomach are unknown.

Small intestinal NGN3+ cells may contribute to both endocrine and non-endocrine lineages

Expression of a β-galactosidase transgene under control of 6.9 kb of flanking sequence of the mouse *ngn3* gene demonstrated that some intestinal endocrine cells may arise from NGN3+ cells. A number of enteroendocrine cells showed β-galactosidase co-localized with chromogranin A despite the absence of *ngn3* expression in these cells (Jenny et al., 2002). The increased stability of the β-galactosidase reporter probably accounts for this discrepancy, although the failure to observe β-galactosidase staining in a significant fraction of NGN3+ cells (Jenny et al., 2002) may indicate that the transgene did not completely recapitulate the expression of *ngn3*. Likewise, a 5.7-kb fragment of the human *ngn3* gene did not direct expression of a β-galactosidase transgene to all NGN3+ cells, further suggesting that shorter transgenes may not contain all regulatory sequences needed to mimic expression of the endogenous gene. In addition, β-galactosidase expression may be transient, like *ngn3*, and may disappear before some cells differentiate. As a result, use of reporter transgenes is likely to significantly underestimate the cell populations arising from NGN3+ cells with failure to detect the reporter in all enteroendocrine cells or some non-endocrine cells.

In the present work, use of a much larger transgene results in expression that closely recapitulates that of *ngn3*. In addition, recombination-based lineage tracing allows permanent labeling of cells, including those that only very transiently express *ngn3* at the earliest stages of development and differentiation. As a result, we have clearly established that all

small intestinal endocrine cells arise from NGN3+ cells. In addition, we further observed the presence of marked, individual goblet and Paneth cells derived from *ngn3*-expressing cells along with unmarked cells within a single crypt–villus unit, which suggests that a few undifferentiated secretory lineage progenitor cells may express *ngn3* before the segregation of the endocrine and non-endocrine lineages. Our data also indicate that *ngn3* expression rarely occurs in pluripotent cells that are either stem cells or their immediate descendents that give rise to all of the epithelial cells originating from a single crypt. Transient or low-level expression of NGN3 in some immature cells may explain the absence of expression of a *ngn3-lacZ* transgene in non-endocrine cells (Jenny et al., 2002). However, in the present work, even low levels of Cre expression may have been sufficient to trigger recombination and permanent cell marking.

Ngn3 is expressed in heterogeneous populations of immature cells in the GI tract

The marking of non-endocrine descendents of NGN3+ cells in the intestine implies that progenitor cells may express transcription factors important for defining multiple lineages before the segregation of these lineages and cell commitment (Fig. 5). Subsequent cell commitment may depend on the relative level of these transcription factors. Our results suggest that *ngn3* is expressed in a population of relatively undifferentiated cells, which also contribute to the goblet and Paneth lineages. These immature cells may express *ngn3* at a level below the threshold needed for endocrine specification but express Cre at a level sufficient to trigger efficient recombination. Enteroendocrine cells, Paneth cells, and goblet cells are thought to arise from a common progenitor cell population based upon the absence of all three of these cell types in *Math1*-deficient mice (Yang et al., 2001). The presence of *Math1* expression in the intestine of *ngn3* null mice further suggests that Math1 is upstream of NGN3 (Jenny et al., 2002). Also, the observation of increased numbers of goblet cells in the absence of *neurogenin 3* suggests that intestinal secretory precursor cells may adopt an alternate cell fate when endocrine differentiation is blocked (Jenny et al., 2002). Our data also indicate that *ngn3* is expressed rarely in pluripotent cells within the intestinal epithelium (Fig. 5). The rare marking of villi we observed may reflect a dynamic relationship between the stem cell compartment and progenitor cells in the intestine.

Similarly, marking of non-endocrine descendents of NGN3+ cells in the pancreas suggests that *ngn3*, like PTF-p48, is expressed transiently in a heterogeneous population of immature, undifferentiated cells. Analysis of the descendants of the transcription factor PTF1-p48 revealed that most islet and duct cells arose from p48+-expressing cells (Kawaguchi et al., 2002). This was not predicted because PTF1-p48−/− mice failed to develop acinar tissue but were capable of forming endocrine lineages. Because non-endocrine cells in the pancreas differentiate normally in the absence of *ngn3*, it is unlikely that transient expression of *ngn3* influences differentiation of non-endocrine cells.

Single-cell transcript analysis of undifferentiated epithelial cells from the fetal pancreas showed that 14 out of the 60 cells analyzed expressed *ngn3*, suggesting that *ngn3* is expressed in a subset of immature cells (Chiang and Melton, 2003). Gene expression profiling of these immature NGN3+ cells revealed that *ngn3* is expressed in a heterogeneous population of progenitor cells. Four NGN3+ cells did not express other endocrine transcription factors but did express PTF1-p48, suggesting that they could differentiate into either endocrine or non-endocrine cells. Another four cells

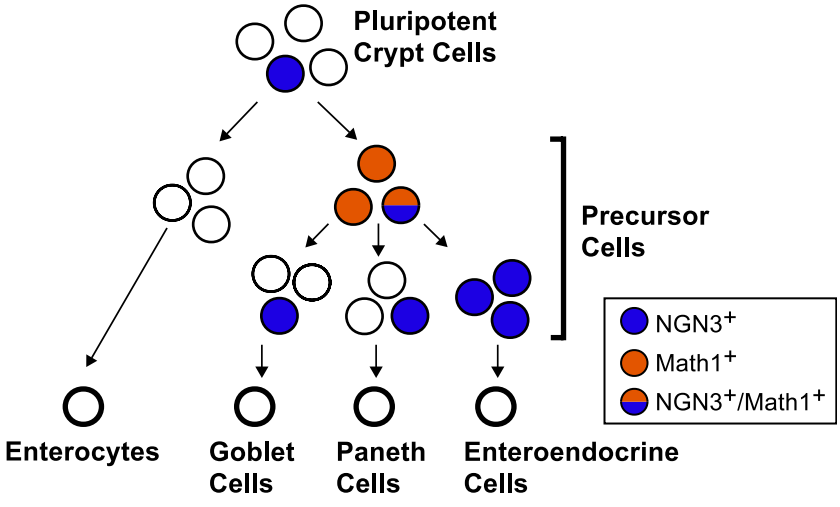

Fig. 5. *Ngn3* is expressed in a mixed population of immature cells. All enteroendocrine cells arise from precursor cells that express *ngn3*. In addition, *ngn3* is expressed in a fraction of Math1+ cells that are progenitors for the three intestinal secretory lineages. Occasional pluripotent crypt cells capable of differentiating into all four intestinal epithelial cell lineages express *ngn3*.

expressed the endocrine transcription factors Pax6 and BETA2/NeuroD as well as PYY, a hormone expressed early in islet differentiation. The remaining six NGN3+ cells also expressed insulin and glucagon, indicative of more advanced endocrine differentiation (Chiang and Melton, 2003). These results and our own finding support the idea that lineage markers may be expressed before cell fate commitment, a phenomenon that has also been observed in the hematopoietic system and has been interpreted to represent a transcriptional "priming" that occurs as progenitors are making cell fate choices (Miyamoto et al., 2002).

In summary, our results, using cell marking techniques, establish that the pro-endocrine gene, *neurogenin 3*, is expressed in precursors of the endocrine cell lineage in the pancreas, the adult intestine, and some precursors in the stomach, and that *ngn3* functions cell autonomously in progenitor cells to direct endocrine specification. This work reveals important similarities and differences in how endocrine cells differentiate throughout the gastrointestinal tract. In addition, the identification of non-endocrine cells that arose from *ngn3*-expressing progenitors suggests that expression of *ngn3* is not sufficient to commit cells to an endocrine cell fate and that allocation of undifferentiated cells to an endocrine progenitor pool is a dynamic, multifactorial process.

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