Neurogenin3 is differentially required for endocrine cell fate specification in the intestinal and gastric epithelium

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Endocrine cells of the pancreas and the gastrointestinal tract derive from multipotent endodermal stem cells. We have shown previously that the basic helix–loop–helix (bHLH) transcription factor neurogenin3 (*ngn3*) is required for the specification of the endocrine lineage in uncommitted progenitors in the developing pancreas. We investigate herein the expression and the function of *ngn3* in the control of endocrine cell development in the intestinal and gastric epithelium. Our results indicate that as in the pancreas, gastrointestinal endocrine cells derive from *ngn3*-expressing progenitors. Mice homozygous for a null mutation in *ngn3* fail to generate any intestinal endocrine cells, and endocrine progenitor cells are lacking. The other main intestinal epithelial cell types differentiate properly. In contrast, in the glandular stomach, the differentiation of the gastrin- (G cells) and somatostatin (D cells)-secreting cells is impaired whereas serotonin- (enterochromaffin EC cells), histamine- (enterochromaffin-like ECL cells) and ghrelin (X/A cells)-expressing cells are still present. Thus, *ngn3* is strictly required for endocrine cell fate specification in multipotent intestinal progenitor cells, whereas gastric endocrine development is both *ngn3* dependent and independent.

Keywords: bHLH/enteroendocrine differentiation/ gastrointestinal tract/neurogenin/progenitor cell

Introduction

Endocrine cells of the digestive tract include pancreatic cells clustered in the islets of Langerhans (Yamaoka and Itakura, 1999) and scattered cells distributed throughout the digestive epithelium, from the stomach to the colon (Hocker and Wiedenmann, 1998), known as enteroendocrine cells. Despite the fact that the relative percentage of endocrine cells is low within the gastrointestinal epithelium, quantitatively enteroendocrine cells constitute the major endocrine organ of the organism. The various

peptide hormones produced by these endocrine cells control important physiological functions such as glycemia, exocrine pancreatic secretion, growth and repair of the gut epithelium, motility of the gut wall and gastric emptying (Skipper and Lewis, 2000). In mammals, the gastrointestinal epithelial cells continue to proliferate actively throughout life. In the small intestinal mucosa, enteroendocrine cells, as well as the three other principal cell types of the gut (enterocytes, goblet cells and Paneth cells), arise from a multipotent stem cell located near the base of the crypts of Lieberkühn (Gordon *et al.*, 1992). It is thought that progenitor cells, located in the proliferative compartment, derive from these stem cells and, while leaving the crypts, stop dividing and differentiate further into each of the intestinal cell lineages during their migration to the villus tip (Booth and Potten, 2000; Marshman *et al.*, 2002). Paneth cells migrate downwards to the base of the crypts. The intestinal enteroendocrine cells consist of at least 15 different cell types classified essentially on the basis of their hormonal content with a specific geographical distribution (Hocker and Wiedenmann, 1998). The stomach epithelium is organized in invaginations known as gastric glands, and it is thought that presumptive stem cells located in a central position in the isthmus region of the gland give rise to all the gastric epithelial cell types (Karam *et al.*, 1997). A majority of the newly formed enteroendocrine cells migrate to the base of the gastric gland (Karam and Leblond, 1993). Transgenic studies have provided important insights into the potential lineage relationship between the different enteroendocrine cell types (Roth *et al.*, 1990; Roth and Gordon, 1990; Lopez *et al.*, 1995; Rindi *et al.*, 1999). However, the early steps of the specification of the endocrine cell lineages in the digestive tract are poorly understood. In particular, the molecular mechanisms controlling endocrine commitment in multipotent stem/progenitor cells in the embryonic as well as the adult intestine and stomach remain to be clarified.

Proteins of the basic helix–loop–helix (bHLH) family are important transcriptional regulators of cell fate determination and differentiation in a number of cell types in both invertebrate and vertebrate species (Hassan and Bellen, 2000; Massari and Murre, 2000). For example, the products of the proneural genes *neurogenin 1* and *2* are bHLH transcription factors required for neural precursor determination in the peripheral and central nervous system (Fode *et al.*, 1998, 2000; Ma *et al.*, 1999). We and others showed recently that the third member of the *neurogenin* family, *neurogenin3* (*ngn3*), is transiently expressed in endocrine progenitors during pancreas development (Apelqvist *et al.*, 1999; Gradwohl *et al.*, 2000; Jensen *et al.*, 2000a; Schwitzgebel *et al.*, 2000). Mice lacking *ngn3* die postnatally from diabetes because they fail to generate any islet cells, and endocrine progenitors are

Fig. 1. *ngn3* is expressed in the embryonic and the adult crypt intestinal epithelium. (A–E) Cryosections through the intestines were hybridized with a *ngn3* riboprobe (dark blue) and double stained (E) with anti-BrdU antibodies (brown). (F) Immunohistochemical staining for *ngn3* protein. (A) *ngn3* transcripts are found in scattered cells from the embryonic stage E12.5 onwards in the developing intestine. (B–D) The number of *ngn3*⁺ cells increases at E14.5–E15.5, and *ngn3*-expressing cells are distributed from the duodenum (C) to the colon (arrowheads in D). (E and F) In the adult small intestine, *ngn3* transcripts (arrowheads in E) and protein (arrows in F) are found exclusively in the proliferative compartment of the crypts mainly in BrdU⁺ cells (E) and never in the villi (F). i, intestine; p, pancreas.

missing (Gradwohl *et al.*, 2000). These results, together with gain-of-function studies (Apelqvist *et al.*, 1999; Grapin-Botton and Melton, 2000; Schwitzgebel *et al.*, 2000), suggest that *ngn3* controls endocrine fate decisions in multipotent pancreatic endodermal progenitors. Endocrine cell development in the intestinal epithelium is also regulated by members of the bHLH transcription factor family. For example, in the mouse intestine, the loss of *Math1* leads to depletion of the secretory cell lineages, including Paneth, goblet and enteroendocrine cells (Yang *et al.*, 2001). In addition, the bHLH protein BETA2/NeuroD has been shown to control terminal differentiation of the enteroendocrine secretin-producing cells by coordinating transcription of the secretin gene with cell cycle arrest (Naya *et al.*, 1997; Mutoh *et al.*, 1998; Ratineau *et al.*, 2002). In contrast, the bHLH transcription factor HES1 negatively regulates enteroendocrine differentiation (Jensen *et al.*, 2000b).

In the present study, we investigated the expression and function of *ngn3* in the developing and adult intestine. We also generated transgenic mice to characterize the activity of the *ngn3* promoter, and took advantage of the stability of the reporter protein to trace *ngn3* progeny in the gastrointestinal tract. Our results indicate that *ngn3* is

expressed in enteroendocrine progenitors all along the proximo-distal axis of the developing intestine and in the adult crypts. No endocrine cells are found in the intestinal epithelium of *ngn3*-deficient mice, and endocrine progenitors are lacking. Based on these results, we propose that, similarly to the developing pancreas, *ngn3* specifies an endocrine fate in uncommitted cells of the intestinal mucosa during both embryogenesis and adult life. In the stomach, enteroendocrine differentiation is both *ngn3* dependent and independent.

Results

ngn3 is expressed in isolated cells in the embryonic gut endoderm and subsequently in the intestinal crypts

The *ngn3* spatio-temporal expression pattern in embryonic and adult mouse intestines has been investigated by *in situ* hybridization (ISH; Figure 1A–E). *ngn3*-expressing cells are first found as early as E12.5 days post-coitum (d.p.c.) in scattered cells within the gut endoderm (Figure 1A). *ngn3* transcripts precede the expression of *NeuroD*, which is detected in the intestinal epithelium from E14.5 d.p.c. onwards (not shown). The number of *ngn3*⁺ cells peaks at E14.5–E15.5, and labeled cells are found from the duodenum to the colon (Figure 1B–D) in a decreasing gradient reflecting the rostro-caudal morphogenesis of the gut. In the adult small intestine, *ngn3*-expressing cells are located exclusively in the proliferative compartment, the crypts (Figure 1E). Double labeling experiments showed that *ngn3* transcripts are found in dividing bromodeoxyuridine-positive (BrdU⁺) cells (Figure 1E; arrowheads) but we also observed a few *ngn3*⁺/BrdU[−] cells in the crypts (data not shown). *ngn3*⁺ cells were not detected in the villi and do not co-stain with chromogranin A, a general marker for differentiated endocrine cells; in contrast, *NeuroD*-expressing cells are found mainly in the villi and co-express chromogranin A (data not shown). Immunohistochemistry with an anti-*ngn3* antibody indicated that *ngn3* protein is also restricted to crypt cells (Figure 1F; arrows).

Intestinal enteroendocrine cells derive from *ngn3* progenitors

bHLH genes are transiently expressed in progenitor cells; their expression is turned off as the cells become post-mitotic and differentiate. Therefore, it is difficult to determine the identity of the cell types derived from these progenitor cells. One strategy to identify the progenitor descending cells is to use a reporter gene coding for a protein more stable than the endogenous bHLH protein. This approach has been used to define the interneurons derived from *Math1* progenitors (Helms and Johnson, 1998) and validated in a *LacZ* knock-in of *Math1* (Birmingham *et al.*, 1999; Ben Arie *et al.*, 2000). Here, we characterize the activity of the mouse *ngn3* promoter in the gastrointestinal tract of transgenic mice and take advantage of the stability of the β -galactosidase protein (Smith *et al.*, 1995) to define the cells deriving from *ngn3*-expressing progenitors.

We generated transgenic mice expressing a nuclear *LacZ* reporter gene under the control of 6.9 kb of 5' genomic sequences. In recent cell lineage tracing

Fig. 2. Intestinal activity of the *ngn3* promoter. The β -galactosidase protein marks *ngn3* progenitor cells and their deriving enteroendocrine cells. A transgenic mouse model where a nuclear *LacZ* is driven by *ngn3* regulatory sequences was generated and the stability of the β -galactosidase protein was used to trace the progeny of *ngn3*-expressing cells. (A–D) Whole-mount X-Gal-stained E15.5 digestive tract (A) and adult duodenum (B) were sectioned (C and D, respectively); the blue nuclear staining indicates β -galactosidase catalysis of the X-Gal substrate. At E15.5, X-Gal-stained cells are found all along the proximo-distal axis of the gut (A) in the intestinal epithelium (C). (E) Immunofluorescent co-staining for β -galactosidase (red) and *ngn3* (green) shows partial overlapping expression (yellow and orange cells, arrows in E). In the adult intestine, β -Gal-labeled cells are found in the crypts (D, red immunofluorescence in F), in dividing PCNA⁺ cells (arrow in F) and also in the villi (D) where they co-stain with the pan-endocrine marker chromogranin A (dark brown cytoplasmic peroxidase staining, arrows in G) in differentiated enteroendocrine cells. The black line in (D) divides the crypt from the villus region. vi, villus; cr, crypt; d, duodenum.

experiments, a very similar *ngn3* promoter fragment has been used to drive the phage CRE recombinase and successfully tag the *ngn3* progeny in the pancreas (Gu *et al.*, 2002). We obtained three founder mice with an identical expression pattern of the transgene. Similarly to the *ngn3* transcript and protein, β -galactosidase activity has been detected all along the proximo-distal axis of the developing intestines (Figure 2A, C and E) as well as in the adult crypts (Figure 2D and F) in dividing progenitors (Figure 2F, arrow). These results indicate that the tested *ngn3* regulatory sequences faithfully recapitulate endogenous gene expression in the gut as well as in the pancreas (Figure 2A; data not shown). The β -galactosidase

is only present in a subset of *ngn3*-expressing cells (Figure 2E, arrows) which is likely to be due to the mosaic expression of transgenes and/or the differences in the maturation and degradation of the two gene products. A similar observation has been reported in the analysis of the human *ngn3* promoter (Lee *et al.*, 2001). In addition, we observed β -Gal⁺ cells in the villi of the adult intestinal epithelium (Figure 2B, D and G) where they co-stain with the pan-endocrine marker chromogranin A (Figure 2G). In contrast, the *LacZ* transcript was found exclusively in the crypt compartment (data not shown). Therefore, the presence of the β -galactosidase in differentiated endocrine cells is not due to an ectopic expression of the transgene but results from the persistence of the *LacZ* gene product in the enteroendocrine lineage although *ngn3* transcription is extinguished. Our data thus demonstrate that intestinal enteroendocrine cells derive from *ngn3*-expressing progenitors.

Intestinal endocrine cells do not develop in *ngn3*-deficient mice

The function of *ngn3* in the development of the intestine and in particular in the differentiation of the enteroendocrine lineage was analyzed in mice homozygous for the *ngn3* deletion that we generated previously. No gross morphological abnormalities have been observed in the digestive tract of *ngn3*-deficient newborn animals, except that they had a smaller stomach (Figure 3A and A'). We also recorded that milk often stagnates in the intestine of the mutant mice (Figure 3A'). To determine whether endocrine cells are present in the mutant intestinal epithelium, an immunocytochemical analysis was performed with antibodies against chromogranin A and the principal intestinal hormones. Endocrine cells can be detected in the wild-type developing gut starting from E15.5 d.p.c. Cells expressing chromogranin A are found in the wild-type small (Figure 3B) and large (not shown) intestine at birth, whereas they could not be detected all along the proximo-distal axis of the intestine (Figure 3B'; data not shown) in the absence of *ngn3*. In addition, the principal intestinal hormones, cholecystokinin (CCK), secretin, gastrin, serotonin, peptide YY (PYY), glucagon-like protein (GLP), gastric inhibitory protein (GIP) and somatostatin are not produced in *ngn3* homozygous intestine (exemplified in Figure 3C' and D'; data not shown) compared with the controls (Figure 3C and D; data not shown). *ngn3* mutant mice die shortly after birth; we could thus have missed delayed entero-endocrine differentiation because intestinal maturation proceeds during the 3 weeks of postnatal life. This possibility was investigated by grafting 12-day fetal intestinal anlagen under the skin of nude mice to rescue the lethal pancreatic phenotype and allow development beyond postnatal day 1. Intestinal grafts were recovered after 4 weeks; they formed vascularized and well developed structures composed of intestinal villi, as well as the mucosal, submucosal and muscular layers (not shown). No endocrine cells could be found in the crypt and villus epithelium of the grafts analyzed, as assessed by the absence of chromogranin A and of hormone (gastrin/CCK, serotonin, GIP, PYY and GLP)-expressing cells, compared with the wild-type intestinal grafts (Figure 3E and E'; data not shown). These data suggest that all the intestinal enteroendocrine

Fig. 3. *ngn3* is required for the differentiation of all the endocrine cell lineages in the intestinal epithelium. The comparison of wild-type (A–E) and *ngn3* knock-out (A'–E') intestines at birth (P₁) (A–D and A'–D') and postnatal stages (E and E') shows that milk often stagnates in the intestine of the *ngn3*-deficient newborns (compare A with A') and that cells expressing the pan-endocrine marker chromogranin A (B) and the endocrine hormones, illustrated for gastrin/CCK (arrowheads in C) and serotonin (arrowheads in D), are observed in wild-type small intestines but are completely missing in *ngn3* mutant intestinal epithelium at birth (compare B–D with B'–D'). Enteroendocrine cells are also missing at later stages of development reached by grafting embryonic intestines under the skin of nude mice and recovered after 4 weeks (E and E'). st, stomach; i, intestine.

cell differentiation, at all time points, is impaired in the absence of *ngn3*.

Enteroendocrine progenitors are lacking in the absence of *ngn3*

Previous studies have shown that members of the bHLH transcription factor family regulate intestinal cell differentiation. *Math1* controls the specification of a common multipotent progenitor for the intestinal secretory cells (goblet, Paneth and enteroendocrine cells), whereas *NeuroD* is known to coordinate terminal differentiation of enteroendocrine cells by inducing cell cycle arrest and activating the transcription of hormone genes. To determine at which stage of enteroendocrine cell development *ngn3* is required, we studied the expression of *Math1* and *NeuroD* in mutant intestinal epithelium. *Math1* transcripts were detected in immature crypt cells but also in differentiated cells in the villi of both wild-type and mutant mice at birth (Figure 4A and A'; arrowheads and arrows, respectively), suggesting that *ngn3* lies downstream of *Math1* in the endocrine lineage. In contrast, *NeuroD* expression is turned off in the absence of *ngn3* (Figure 4B and B'). *ngn3* would thus act upstream of *NeuroD* and control the development of the enteroendocrine progenitor cells, before they leave the cell cycle and differentiate. The loss of *Pax6* expression (Figure 4D), a paired box gene reported to be expressed widely in the enteroendocrine lineage and controlling GIP cell differentiation (Larsson *et al.*, 1998), confirmed the upstream position of *ngn3* in the cascade of transcription factors regulating enteroendocrine differentiation.

To define further the function of *ngn3*, we examined the expression of the *LacZ* reporter gene of *ngn3* promoter::*LacZ* animals in a *ngn3*^{-/-} background (genotype: *ngn3*-promoter::*LacZ*; *ngn3*^{-/-}). Interestingly, we could not detect any β -galactosidase activity in the

intestinal epithelium of mutant animals (Figure 4C and C'), suggesting that enteroendocrine progenitors did not develop in *ngn3* mutant mice.

The major intestinal epithelial cytotypes develop normally in *ngn3*^{-/-} mice

The consequences of the *ngn3* mutation on the overall development of the intestinal mucosa as well as on the differentiation of the other intestinal cell types were examined in both newborn animals and grafted embryonic intestines as described above. Through histological analysis, we did not detect any obvious differences in the development of the mucosal layer, and the crypt and villi organization was not altered in the intestine of mice lacking *ngn3* (Figure 5A and A'). Similarly, the muscular layers, the subepithelial myofibroblasts and the muscle fibers within the villus conjunctive core developed correctly, as determined by the expression of specific markers (data not shown). However, we consistently observed that the thickness of the mucosal and muscular layers was increased, at least in the proximal intestine of *ngn3* homozygous mice at late postnatal stages (data not shown). In contrast to the endocrine cell types, the other epithelial cytotypes differentiate as enterocytes, mucus and Paneth cells. Indeed, differentiation markers of enterocytes, such as lactase activity in newborns (Figure 5B and B') and sucrose activity at later stages (grafts, data not shown), were detected in the intestinal epithelium of control and mutant animals. In addition, the shift between the two digestive enzymes, signified by a decrease in lactase activity and induction of sucrose, occurred in the grafting conditions at stages corresponding to weaning in both knock-out and wild-type intestines, suggesting that the enterocyte lineage differentiated properly in the absence of *ngn3*. Goblet cells were clearly identified in the small and large intestine by the production

Fig. 4. The intestines of *ngn3* mutants lack endocrine progenitors. In wild-type intestines, *Math1* (ISH) is expressed in common multipotent progenitors for secretory cells located in the crypts [arrowheads in (A)] as well as in differentiated cells (arrows in A), whereas *NeuroD* (ISH) is expressed only in post-mitotic enteroendocrine cells (B). *Math1* and *NeuroD* expression are maintained [arrowheads and arrows in (A')] and turned off (B'), respectively, in the absence of *ngn3*, suggesting that *ngn3* acts downstream of *Math1* and upstream of *NeuroD* in the differentiation of the intestinal endocrine lineage. To address the question of the fate of the *ngn3* mutant cells in the gastrointestinal tract, the *ngn3* promoter::LacZ and *ngn3*^{+/-} transgenic mice were crossed to follow the expression of the β-galactosidase transgene in a *ngn3* mutant background. β-Gal⁺ cells [red immunofluorescence, arrowheads in (C)] were absent in the *ngn3*^{+/-} intestines (C'), indicating the early function of *ngn3* in the determination of the endocrine lineage in the developing intestine. (D) RT-PCR analysis of gene expression in P₁ intestine. Expression of Pax6 is lost in *ngn3*-deficient intestine. TBP was used as an internal standard.

of mucus in mice lacking *ngn3* (Figure 5A and A'; data not shown) but, interestingly, their number increased from an average of 363 ± 45 to 585 ± 28 goblet cells/mm² of intestinal mucosa in the P1 small intestine (an average of three wild-type and mutant animals were analyzed). The colon did not show a similar increase in goblet cell density. Finally, Paneth cells were seen in grafted intestines at their expected position in the bottom of the crypts as clusters of cells filled with secretory granules both in *ngn3* homozygous mice and in controls (Figure 5C and C', arrowheads).

Fig. 5. Non-endocrine lineages (enterocytes, goblet and Paneth cells) develop normally in *ngn3*-deficient intestinal epithelium. The development of the intestinal mucosa [compare (A) with (A')] as well as the differentiation of enterocytes [lactase activity in (B) and (B')], goblet cells (PAS staining, arrowheads in (A) and (A')) and Paneth cells [PAS staining, arrowheads in (C) and (C')] pointing to the Paneth cells localized at the bottom of the crypts] are not affected in *ngn3* mutants at birth (A and A', B and B') and at postnatal stages [4 weeks intestinal grafts (C) and (C')], suggesting that *ngn3* does not control the determination of these intestinal cytotypes either directly or indirectly. A 60% increase in the number of goblet cells has, however, been observed in *ngn3*^{+/-} duodenum [compare (A) and (A')].

Gastric endocrine development is unevenly affected in *ngn3*^{-/-} mice

ISH with an antisense *ngn3* riboprobe revealed that *ngn3* transcripts are present in the gastric epithelium at postnatal stages and in the adult (data not shown). As described above for the gut, we took advantage of the β-galactosidase protein stability to determine the identity of the *ngn3*-expressing cells and to trace their progeny in the *ngn3* promoter::LacZ transgenic mice. Whole-mount analysis revealed that the reporter protein is expressed, in addition to the intestine, in the glandular part (oxyntic and antro-pyloric region) of the stomach at postnatal stages (Figure 6A) and in the adult (data not shown). This region corresponds to the area that normally contains gastric endocrine cells. No staining was observed in the proximal third of the stomach (forestomach) which does not contain any glands. To determine if *ngn3* was expressed in the gastric endocrine lineage, the stomach of the transgenic mice was sectioned and double stained for β-galactosidase

activity and chromogranin A, which is expressed in all the gastric endocrine cell types (Norlen *et al.*, 2001). The *LacZ* transgene product was expressed mainly in isolated cells in the gastric epithelium and some of the β -Gal⁺ cells co-stained for chromogranin A (Figure 6B, arrow). To characterize the gastric *ngn3* lineage further, similar double staining experiments have been performed with specific markers for the different gastric endocrine cell types (Solcia *et al.*, 2000). Gastrin (G cells), somatostatin (D cells), serotonin (enterochromaffin EC cells), ghrelin (X/A cells) and histamine [enterochromaffin-like ECL cells expressing the specific marker histidine decarboxylase (Chen *et al.*, 1999)] secreting cell types were all marked by the transgene (Figure 6C–G). These results suggest that *ngn3*, as is the case in the pancreas and the

Fig. 6. *ngn3* promoter activity in the gastric endocrine lineage. (A) *ngn3* regulatory sequences target the expression of the *LacZ* gene (β -galactosidase activity) in the glandular part (g) of the newborn stomach but not in the squamous epithelium (s) of the forestomach. (B–G) Double stainings for β -galactosidase (blue nuclear staining) and chromogranin A [CA in (B)], gastrin [GAS in (C)], somatostatin [SOM in (D)], serotonin [SER in (E)], ghrelin [GHR in (F)] and histidine decarboxylase [HDC in (G)] (brown peroxidase staining), on sections of newborn (B–F) and adult (G) stomachs. β -galactosidase is expressed in progenitors of the gastric endocrine cells as suggested by the high number of single β -galactosidase-expressing cells (blue enzymatic activity) and the persistence of β -galactosidase activity in differentiated endocrine cells (arrows in B–G).

intestine, is also expressed in gastric endocrine progenitors and that these *ngn3* progenitors will give rise to all the principal gastric endocrine cell types.

To determine whether gastric enteroendocrine differentiation was affected in *ngn3*-deficient mice, an immunohistochemical analysis was performed to detect chromogranin A and specific endocrine cell types. Cells positive for chromogranin A were still present in the *ngn3*^{−/−} gastric epithelium (Figure 7A and A'), indicating that endocrine differentiation occurred in *ngn3*-deficient stomach. However, gastrin- and somatostatin-expressing G and D cell differentiation was impaired in mice lacking *ngn3* (Figure 7B' and C') compared with the wild-type gastric mucosa (Figure 7B and C). In contrast, serotonin- and ghrelin-producing EC and X/A endocrine cell types were still present in the mutant gastric epithelium (Figure 7D' and E'). Since ECL cells are not yet present in the mouse oxyntic mucosa at birth (our own observation), to address the role of *ngn3* in ECL cell differentiation we grafted control and *ngn3*^{−/−} stomachs, as described above, to reach postnatal stages. Histidine decarboxylase is detected in both wild-type and mutant grafted stomachs (Figure 7F and F'), suggesting that the differentiation of ECL cells, which represent 65–75% of the gastric endocrine cell population (Chen *et al.*, 1999), is not controlled by *ngn3*. The lack of G and D cells observed at birth in *ngn3* mutant gastric epithelium (Figure 7) has been confirmed in the grafted *ngn3*-deficient stomachs (not shown).

Discussion

The intestinal epithelium is replaced every 3–4 days in rodents by the generation of multiple cell lineages from multipotent epithelial stem cells. The different intestinal cell types probably do not derive directly from this stem cell, rather it is thought that they are produced from an intermediate population of committed progenitor cells (Bjerknes *et al.*, 1999). Recent studies support the hypothesis that the secretory cell lineages (goblet, enteroendocrine and Paneth cells) derive from a common

Fig. 7. Gastric endocrine specification is both *ngn3* dependent and independent. Cells expressing the endocrine marker chromogranin A (CA) (A), and the hormones gastrin (B), somatostatin (C), serotonin (D) and ghrelin (E) are detected by immunohistochemistry in the wild-type gastric mucosa at birth. In *ngn3* mutants, gastrin-secreting G cells (B') and somatostatin-secreting D cells (C') are lost. However, endocrine cells expressing chromogranin A (A'), serotonin (D') and ghrelin (E') are still observed. Histidine decarboxylase (HDC), a specific marker of ECL cells, is expressed in both grafted control (F) and *ngn3*-deficient (F') stomachs at postnatal stages. The arrowhead in (B') points to a rare G cell detected in *ngn3*^{−/−} stomach.

progenitor expressing the bHLH transcription factor Math1 (Yang *et al.*, 2001). From these results, it is reasonable to suggest that the secretory and absorptive lineages arise from two types of progenitors specified on the basis of the expression of the bHLH transcription factor Math1 (Figure 8A). However, very little is known about the genetic programs which control the commitment of the *Math1*-positive multipotent progenitors into the different secretory lineages and in particular the enteroendocrine lineage. Gene targeting experiments demonstrated that the bHLH protein NeuroD/BETA2, which is expressed in all enteroendocrine cells (Naya *et al.*, 1997), is required specifically for the differentiation of the secretin- and CCK-expressing cells (Naya *et al.*, 1997), whereas the remaining enteroendocrine cells develop properly in the absence of NeuroD/BETA2 (Rindi *et al.*, 1999). Other experiments demonstrated that, in the intestine, NeuroD/BETA2 coordinates the transcription of the secretin gene with cell cycle arrest (Mutoh *et al.*, 1998). Thus NeuroD/BETA2 is promoting the terminal differentiation of secretin cells rather than controlling

early steps in the development of the enteroendocrine cell types.

In the present study, we show that another member of the bHLH transcription factor family, *ngn3*, is expressed in the fetal intestinal epithelium as well as in immature cells located in the proliferative compartment of the crypts in the adult small intestine. Differentiated endocrine cells in the villi which express *NeuroD* (Rindi *et al.*, 1999) do not express *ngn3*. However, lineage tracing experiments of the *ngn3* progeny shown herein demonstrate that crypt *ngn3*-expressing cells finally give rise to chromogranin A-positive enteroendocrine cells. This conclusion has been drawn from the analysis of transgenic mice that we produced expressing the *LacZ* gene under the control of *ngn3* regulatory sequences. In these mice, the reporter recapitulates endogenous intestinal *ngn3* expression, and the long half-life of β -galactosidase allowed us to follow the fate of the *ngn3*-expressing cells. Together, these expression data suggest that, as is the case in the developing pancreas, *ngn3* marks an endocrine committed progenitor population, which does not yet express endocrine hormones. Gene targeting experiments showed that all the intestinal endocrine cells fail to develop in *ngn3*^{-/-} mice at embryonic stages, at birth and also at later postnatal stages that could be reached by grafting mutant intestines under the skin of nude mice. This latter experiment demonstrates that early lethality cannot explain the loss of enteroendocrine cells. Importantly, the expression of *NeuroD* is lost in the *ngn3*^{-/-} intestine, whereas *Math1*-expressing cells are still detected in the crypts and the villi. Therefore, we propose that *ngn3* has an early function in the development of the intestinal endocrine lineage, before the cells exit the cell cycle. *ngn3* probably acts in a regulatory cascade downstream of or parallel to *Math1* and upstream of *NeuroD*, and would thus be required for the endocrine fate commitment of a *Math1*⁺ multipotent progenitor of the secretory lineages (Figure 8A). This hypothesis is supported further by the lack of β -galactosidase protein in the embryonic intestine of *ngn3* promoter::*LacZ* mice in a *ngn3*^{-/-} background, which suggests that endocrine progenitors are lacking in the absence of *ngn3*. Similar observations have been made in the pancreas (M.Jenny and G.Gradwohl, unpublished results). One can therefore hypothesize that the development of the enteroendocrine lineage is blocked at the *Math1*-expressing progenitor stage in *ngn3*^{-/-} intestine. However, we cannot completely rule out that some endocrine progenitors are generated but then fail to proliferate. One other possible explanation for the failure of *LacZ* transgene expression in an *ngn3*^{-/-} background could be that *ngn3* gene transcription is auto-regulated. We find that the latter hypothesis is unlikely since β -galactosidase⁺ progenitors can be detected in the *ngn3*-deficient gastric epithelium (data not shown). The common requirement for *ngn3* function in the generation of CCK-, secretin-, gastrin-, GIP-, GLP-, PYY-, serotonin- and somatostatin-producing intestinal cell types suggests that these enteroendocrine lineages originate from a common endocrine progenitor. However, we cannot rule out that the different endocrine cells arise from distinct progenitors that each express *ngn3*. We are inclined to believe in the possibility that *ngn3* acts to specify a common intestinal endocrine progenitor, as several studies

Fig. 8. Proposed model for the role of *ngn3* during endocrine cell differentiation in the gastrointestinal epithelium. (A) *ngn3* is required for the differentiation of all the intestinal endocrine cell types and controls endocrine cell fate commitment of *Math1*-positive multipotent progenitors of the secretory lineages. All the endocrine cell types arise from a common *ngn3*-specified progenitor. *ngn3* could compensate for the absence of *NeuroD* in enteroendocrine cell types, except for CCK- and secretin-producing cells. (B) *ngn3* is essential for the differentiation of G and D cells in the stomach. EC, X/A and ECL cell differentiation occurs in mice lacking *ngn3* although they probably derive from *ngn3* progenitors. Another gene would control the endocrine determination of these three gastric endocrine cell types. The *ngn3*-dependent endocrine cell types of the gastrointestinal tract are in red. CCK, cholecystokinin; SEC, secretin; GAS, gastrin; SER, serotonin; GIP, gastric inhibitory peptide; SOM, somatostatin; GLP, glucagon-like peptide; PYY, peptide YY.

have noted co-expression of certain combinations of intestinal hormones (Lopez *et al.*, 1995; Upchurch *et al.*, 1996; Rindi *et al.*, 1999).

Although none of the other main intestinal cytotypes displayed altered differentiation characteristics, an interesting observation is that we found an increase in the number of goblet cells in *ngn3*^{-/-} small intestine at birth. This suggests, but does not prove the existence of a bipotential progenitor (endocrine/goblet). In the absence of *ngn3*, endocrine commitment is no longer possible and the cells would then adopt the alternative goblet fate. This hypothesis that enteroendocrine and goblet cells might share part of their developmental program is supported further by the increase of goblet cells observed in *Hes1* knock-out mice (Jensen *et al.*, 2000b) and the reported switch of pure enteroendocrine cells to a more goblet-like phenotype in Crohn's disease (Poulsom *et al.*, 1993). One alternative explanation could also be that the increase in goblet cells is due to their filling up of a compartment that would otherwise be filled by an expanding population of endocrine cells. The high number of goblet cells in the colon (major cell type) could explain why such an increase was not observed in the colonic crypts or suggest that goblet cell regulation might be different in the colon and the small intestine.

Thus, the data presented here provide evidence that *ngn3* is a determination gene which controls an endocrine fate decision in multipotent intestinal progenitors. In addition, our results demonstrate that *ngn3* specifies the intestinal endocrine lineage in a cell-autonomous manner since we proved that enteroendocrine cells derive from *ngn3*-expressing cells. Other as yet unidentified bHLH genes might specify the fate of the other intestinal cytotypes. Our results are consistent with a previous report suggesting that the Notch–Hes signaling system is involved in endodermal endocrine fate determination in the developing gut (Jensen *et al.*, 2000b). Indeed, Jensen *et al.* observed an increase of all endocrine populations and an up-regulation of *ngn3* and *NeuroD* in the gut endoderm of mice lacking the bHLH repressor gene *Hes1*. Together with our findings, one can hypothesize that the positive regulation of *ngn3* on enteroendocrine cells is antagonized by Hes-1 in neighboring cells upon the activation of the Notch receptor by Delta ligand and subsequent up-regulation of *Hes1*.

The intestinal and pancreatic endocrine phenotype of the *ngn3* mutation prompted us to analyze endocrine differentiation in the stomach, another endodermally derived tissue containing a number of different endocrine cell types (Solcia *et al.*, 2000). We showed that gastrin- and somatostatin-producing G and D cell differentiation is impaired in the stomach of mice lacking *ngn3* (Figure 8B). However, we observed that a significant number of serotonin-expressing enterochromaffin EC cells were still present in the *ngn3*^{-/-} gastric epithelium. While this manuscript was in preparation, Lee *et al.* (2002) reported a similar observation and showed in addition that *ngn3* is important for the maintenance of gastric epithelial cell identity. Our studies extend these findings by demonstrating that the differentiation of two additional gastric endocrine cell types is not affected in *ngn3* mutant mice: the X/A cells producing ghrelin (Date *et al.*, 2000), a novel growth hormone secretagogue and anorexigenic peptide

(Wang *et al.*, 2002), and the ECL cells secreting histamine which play a key role in the regulation of gastrin-stimulated acid secretion (Chen *et al.*, 1999). ECL cells are not detected at birth when *ngn3* mutants die; therefore, the differentiation of this particular cell type could only be studied in rescued intestines. However, the persistence of the *ngn3* promoter-driven β -galactosidase in all the differentiated gastric endocrine cell types studied suggests that although *ngn3* does not control endocrine differentiation of serotonin- (EC), ghrelin- (X/A) and histamine- (ECL) producing cells, they derive from *ngn3*-expressing progenitors. Interestingly, serotonin-producing cells are present both in the small intestine and in the antral mucosa of the stomach. Although these cells secrete the same hormone in both tissues and derive from *ngn3*-expressing progenitors, they are differentially affected in the gut (lost) and the stomach (retained) in the absence of *ngn3*. Thus the differentiation of similar endocrine cell types is controlled by different genetic programs in the gut and the stomach. Similar observations have been made in the ventral telencephalon where there are some GABAergic neurons which express the bHLH transcription factor *Mash1* which are *Mash1* independent while others are dependent (Casarosa *et al.*, 1999).

In conclusion, our results indicate that *ngn3* is expressed specifically in endocrine progenitors of the developing and adult intestine as well as of the stomach. Our loss-of-function experiment demonstrates that *ngn3* is required for the specification of the endocrine fate in multipotent intestinal progenitors (Figure 8A) and that endocrine differentiation is completely impaired in the mutant intestine. In contrast, gastric endocrine differentiation does not rely entirely on *ngn3* function. As a consequence, at least two different gastric endocrine lineage pathways exist, one dependent on and the other independent of *ngn3*, and controlled by an as yet unidentified gene (Figure 8B). Taken together, the work described here and our previous data demonstrate that endocrine cell fate determination is similar in the intestine and the pancreas, but different in the stomach. These findings on the common mechanisms occurring in the gut and the pancreas may initiate the development of novel strategies to derive insulin-secreting β cells from intestinal endocrine progenitors.

Materials and methods

Transgene construction and *ngn3*-deficient mice

To generate the *ngn3* promoter::LacZ construct, a 6.86 kb *XbaI*–*XhoI* fragment (6696 bp of 5' genomic and untranslated region sequences and 176 bp of *ngn3* coding region) of mouse *ngn3* genomic DNA (Gradwohl *et al.*, 2000) was cloned upstream of the IRES-NLS-LacZ-pA sequence in pBS-INL vector (Fode *et al.*, 2000), resulting in plasmid p*ngn3*(6.8)-INL. The 10.6 kb *ngn3*–LacZ insert was released by *NotI* digestion and microinjected into murine oocyte pronuclei, and three independent transgenic lines were generated and maintained by crossing into a CD1 outbred background. Transgenic progeny were identified by PCR using primer in the LacZ gene, VW233 5'-GCACATCCCCCTTTCGCCAG-CTGGCGTAAT-3' and VW234 5'-CGCGTCTGGCCTTCCTGTAGCC-AGCTTTCA-3'. *Ngn3*^{-/-} animals were generated as described previously (Gradwohl *et al.*, 2000).

Multiplex RT-PCR

Multiplex RT-PCR was performed on dissected P1 duodenum, jejunum and colon as described by Jensen *et al.* (2000b). TBP (encoding TATA-binding protein) was co-amplified as an internal standard.

β-galactosidase and lactase activity detection

Tissues were fixed for 10–20 min at room temperature in 0.2% glutaraldehyde, 5 mM EGTA pH 7.3, 2 mM MgCl₂ in 0.1 M sodium phosphate pH 7.3, washed three times for 10 min in LacZ wash buffer (2 mM MgCl₂ in 0.1 M sodium phosphate pH 7.3, 0.02% NP-40) and whole mount staining was performed in a solution containing 1 mg/ml X-Gal, 5 mM potassium ferrocyanide and 5 mM potassium ferricyanide in LacZ wash buffer at 37°C for 2–4 h. After staining, samples were washed in phosphate-buffered saline (PBS), post-fixed with Bouin and processed for wax sections. For double labeling experiments, X-Gal stainings were realized directly on paraformaldehyde-fixed frozen tissue sections, followed by immunostaining. Enterocyte lactase activity was revealed as detailed in Jost *et al.* (1998).

RNA in situ hybridization (ISH), immunohistochemistry and immunofluorescence

RNA ISH experiments were performed on frozen paraformaldehyde-fixed tissues sections as described previously (Cau *et al.*, 1997; Gradwohl *et al.*, 2000). In some cases, ISH was followed by immunostaining. The following cRNA probes were used: *ngn3* (Gradwohl *et al.*, 2000), *math1* (kindly provided by R.Kageyama, Kyoto University, Japan) and *NeuroD* (Fode *et al.*, 1998). Immunohistochemistry and immunofluorescence were performed on paraffin and cryosections as described previously (Cau *et al.*, 1997). The following antibodies were used: rabbit anti-chromogranin A at 1:300 (Diasorin), guinea pig anti-*ngn3* at 1:1000 (kindly provided by M.German, UCSF, San Francisco, CA), rabbit anti-β-galactosidase 1:500 (ICN), rabbit anti-somatostatin at 1:200 (Dako), rabbit anti-CCK/gastrin at 1:750 (INSERM U.45, 8E), rabbit anti-serotonin at 1:5000 (Incstar) or mouse anti-serotonin at 1:50 (Dako), rabbit anti-secretin at 1:2000 (kindly provided by P.Robberecht, Brussels, Belgium, 13/4), rabbit anti-GIP at 1:500 (INSERM U.45, 0.59A), rabbit anti-PYY at 1:1000 (INSERM U-45, A4D), rabbit anti-ghrelin at 1:2000 (kindly provided by C.Tomasetto, IGBMC, Strasbourg, France), rabbit anti-GLP at 1:1000 (INSERM U.45, 199D), rabbit anti-HDC at 1:800 (Progen) and mouse anti-proliferating cell nuclear antigen (PCNA) at 1:100 (Dako). Secondary antibodies used were: Alexa 488 anti-rabbit at 1:500 (Molecular probes), Cy3 anti-guinea pig at 1:500 (Jackson immunoresearch), and peroxidase-coupled anti-rabbit, anti-guinea-pig and anti-mouse at 1:200 (Vector Laboratories). BrdU incorporation and detection experiments were as described by Parras *et al.* (2002). Goblet cells were stained for mucin using the PAS reaction.

Grafting experiments

To analyze *ngn3* knock-out tissues beyond postnatal day 1, 12 day fetal intestine and stomach dissected out from *ngn3*^{-/-} and wild-type mice were grafted under the skin of nude mice. Fetal intestines were subdivided into four parts corresponding to the presumptive duodenum, jejunum, ileum and colon. The intestinal grafts were recovered after 4 weeks, at a stage corresponding to weaning. Grafted stomachs were recovered after 2–3 weeks, because the gastric mucosa suffered from longer development. A mean of three grafts developed from each proximo-distal intestinal segment and from the stomach were analyzed.

Acknowledgements

We thank Dr Andrew Leiter for critical reading of the initial version of the manuscript, Christiane Arnold, Caroline Daurat and Viviane Hauer for excellent technical assistance, Drs Catherine Tomasetto, Rolf Hakanson and Lo Persson for helpful discussions, Drs Mike German and Ryoichi Kageyama for providing us with the *ngn3* antibody and the *Math1* cDNA, respectively, and Marianne LeMeur, Elisabeth Metzger and all the members of the mouse facilities of the IGBMC for their help in the generation of the *ngn3*-promoter::LacZ transgenic mice. This work was supported by funds from the Institut National de la Santé et de la Recherche Médicale (INSERM; AVENIR grant to G.G.), the Ministère de la Recherche et de la Technologie (ACI Biologie du développement et Physiologie Intégrative 2000) and grants from the Association pour la Recherche sur le Cancer (ARC), the Juvenile Diabetes Research Foundation (JDRF; 4-2001-434) and National Institutes of Health (NIH; 1U19-DK61244-01) to G.G. M.J. is a recipient of PhD studentship from the Ministère de la Recherche et de la Technologie.

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Received August 22, 2002; revised October 14, 2002;
accepted October 16, 2002