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Minireview: Development and Differentiation of Gut Endocrine Cells

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For over 30 yr, it has been known that enteroendocrine cells derive from common precursor cells in the intestinal crypts. Until recently, relatively little was understood about the events that result in commitment to endocrine differentiation or the segregation of over 10 different hormone-expressing cell types in the gastrointestinal tract. The earliest cell fate decisions appear to be regulated by the Notch signaling pathway. Notch is inactive in endocrine precursor cells, allowing for expression of the proendocrine basic helix-loop-helix proteins Math1 and neurogenin3. Differentiating precursor cells activate Notch in neighboring cells to switch off expression of proendocrine factors and inhibit endocrine differentiation. Math1 is the first factor involved in endocrine specification, committing cells to become one of three secretory lineages—goblet, Paneth, and enteroendocrine. Neurogenin3 appears to be a downstream target that is essential for endocrine cell differentiation. Events that control the segregation of each mature lineage from progenitor cells have not been characterized in detail. The transcription factors Pax4, Pax6, BETA2/NeuroD, and pancreatic-duodenal homeobox 1 have all been implicated in enteroendocrine differentiation. BETA2/NeuroD appears to coordinate secretin gene expression in S-type enteroendocrine cells with cell cycle arrest as cells terminally differentiate. Powerful genetic approaches have established the murine intestine as the most important model for studying enteroendocrine differentiation. Enteroendocrine cells in the mouse are remarkably similar to those in humans, making it likely that insights learned from the mouse may contribute to both our understanding and treatment of a variety of human disorders. (*Endocrinology* 145: 2639–2644, 2004)

ENDOCRINE CELLS WITHIN the gut epithelium from the stomach to the colon represent the largest population of hormone-producing cells in the body (1). Endocrine cells are scattered as individual cells throughout the mucosa, comprising approximately 1% of the cells lining the intestinal lumen (2). Thus, unlike many endocrine glands, gastrointestinal endocrine cells differentiate in tissues where the overwhelming majority of surrounding cells are nonendocrine, including enterocytes, goblet cells, and Paneth cells in the intestine as well as parietal cells, chief cells, and mucous neck cells in the stomach. This review will focus on our current understanding of the development and differentiation of gut endocrine cells.

With the emergence of immunohistochemical techniques in the 1960s, gastrointestinal endocrine cells were found to express markers for neuronal differentiation, including those involved in the biosynthesis of neurotransmitters, as well as showing ultrastructural properties common to neurons. As a result, gut endocrine cells were classified as APUD cells, leading to the hypothesis that enteroendocrine cells arose from cells that migrated from the neural crest to the gut (3). Embryonic cell tracing techniques have clearly established that gastrointestinal endocrine cells are derived from the endoderm and not the neuroectoderm (4–8). However, the original observations that enteroendocrine cells share features with neurons has assumed new significance with recent discoveries showing that gut endocrine differentiation is regulated similarly to differentiation in the nervous system. As will be discussed later, both neuronal and gut endocrine differentiation appear to be controlled by similar and in some cases identical genes encoding basic helix-loop-helix (bHLH) transcription factors under control of the Notch signaling pathway. Many of the breakthroughs in understanding how these molecules function during development and in endocrine differentiation were discovered using genetic mouse models and will be a major focus of this review. Virtually all of the transcription factors and signaling molecules discussed in this review are highly conserved, particularly between mammals. Furthermore, experiments in human cell lines, expression profiles for some of these factors in human tissue, and the use of human homologs in functional experiments clearly suggests that endocrine differentiation and development is highly conserved between mice and humans. Finally, gene mutations that cause defects in murine endocrine differentiation in the pancreas and intestine have been associated with human disease. For example, human mutations in one allele of BETA2 have been associated with late-onset diabetes, pancreatic-duodenal homeobox 1 (Pdx1) with MODY (maturity onset diabetes of the young), and Pax6 with aniridia and diabetes (9–11).

Gut Endocrine Cells Arise from Multipotential Progenitors

All four epithelial cell types of the intestine, including enteroendocrine cells, differentiate from common pluripotent stem cells in the crypt compartment of the intestine (2). Tritiated thymidine labeling after continuous infusion of the isotope revealed that cells deep in the crypt labeled first,

Abbreviations: bHLH, Basic helix-loop-helix; GLP, glucagon-like peptide; HES, hairy/enhancer of split; NGN3, neurogenin 3; NICD, Notch intracellular domain; Pdx1, pancreatic-duodenal homeobox 1.

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followed by more differentiated cells as one moved out of the crypt, including enterocytes, goblet cells, enteroendocrine cells, and Paneth cells. Cell-labeling kinetics indicate that enterocytes, goblet cells, and enteroendocrine cells differentiate as cells migrate up the crypt-villus axis turning over every 3–4 d, whereas Paneth cells migrate downwards turning over more slowly (2). Unlike many endocrine cells in different glands that differentiate early in life and turnover slowly, enteroendocrine cells actively self-renew and differentiate throughout the life of an animal from a large reservoir of stem cells. As mature enteroendocrine cells migrate to the tips of the villi, they presumably undergo apoptosis and are extruded into the lumen (12).

How stem cells are allocated to differentiate into endocrine cells is not completely understood. For many years, it was not known whether each endocrine cell type differentiated from its own precursor, or whether all enteroendocrine cells segregate from a common progenitor cell. The first clues came from the identification of individual cells coexpressing more than one hormone. In the small intestine, a subpopulation of substance P-expressing cells in villi coexpressed serotonin and secretin, whereas proliferating substance P cells in the crypts did not, leading to speculation that serotonin and secretin cells arise from substance P cells (13, 14). In the colon, peptide YY, glucagon-like peptide (GLP)-1, cholecystokinin, and neurotensin are coexpressed in some cells. Likewise, serotonin-expressing cells often coexpress substance P but never the preceding four hormones, leading to the hypothesis that there are two major branches for enteroendocrine differentiation in the colon (15).

Expression of stable reporter genes in transgenic mice suggests that enteroendocrine cells differentiate from multipotential progenitor cells. Expression of a human GH reporter under control of the liver-fatty acid binding protein (L-FABP) gene showed labeling of multiple lineages of enteroendocrine cells (13). Similarly a secretin-human GH transgenic mouse showed that the transgene was coexpressed in several enteroendocrine cell types in addition to secretin cells, suggesting that multiple lineages share a developmental relationship (16). Another approach for establishing a common origin involves the expression of a toxic gene in specific cell types of transgenic mice. Expression of herpes simplex virus 1 thymidine kinase in secretin-expressing cells of transgenic mice rendered cells susceptible to the antiviral drug ganciclovir. Treatment of transgenic mice with the drug resulted in depletion of secretin cells as well as most cells expressing cholecystokinin and L cells expressing peptide YY and GLP-1. In addition, the numbers of cells expressing serotonin, gastric inhibitory polypeptide (GIP), substance P, and somatostatin were significantly reduced as well, revealing previously unappreciated relationships between these different cell types and secretin cells. The results further suggested that many cell lineages express secretin at levels below the detectable limit for immunostaining. The enteroendocrine cells repopulated the small intestine in normal numbers after withdrawal of ganciclovir, indicating that stem cells were not targeted (17).

Role of Notch Signaling in Enteroendocrine Differentiation

Signaling by the cell surface protein, Notch, plays a critical role in endocrine cell fate determination in the intestine. Notch proteins mediate cell fate decisions and patterning in different tissues of invertebrates and vertebrates by regulating expression of bHLH transcription factors that control terminal differentiation (18). One of the functions of Notch signaling is to mediate lateral inhibition between adjacent cells in a field of initially identical cells; therefore, the first cell that begins differentiation prevents the neighboring cells from adopting the same cell fate (19, 20). This mechanism of inhibition produces specialized cell types that are scattered in appearance, similar to how endocrine cells appear in the gastrointestinal tract. Analysis of transgenic mice with mutations that disrupt Notch signaling indicates that Notch regulates enteroendocrine differentiation by inhibiting expression of proendocrine bHLH transcription factors in the gastrointestinal tract (21, 22).

Gene inactivation studies in mice have identified three *atonal*-related bHLH factors important for intestinal endocrine differentiation—Math1, neurogenin3 (NGN3), and BETA2/NeuroD (BETA2). These factors are structurally related to the *Drosophila atonal* gene, which is important in neural differentiation and contain two amphipathic helices that act in dimerization and a basic domain which directs DNA binding (for review see Ref. 23). This family of transcriptional regulators functions in cascades, where one factor activates a later factor and continues in a sequential order to control both cell fate determination and differentiation of specific cell types. Math1, NGN3, and BETA2 appear to be expressed sequentially during the course of neuronal differentiation, emphasizing the importance of earlier work showing similarities between neurons and enteroendocrine cells (24–26). As will be described later, the sequential appearance of Math1, NGN3, and BETA2 may represent distinct stages in the differentiation of enteroendocrine cells. Therefore, regulation of early-acting bHLH factors by Notch signaling may regulate the earliest stage of cell fate determination of the endocrine lineage, whereas late-appearing factors control differentiation of a specific cell type.

Notch proteins comprise a family of four transmembrane receptors in mammals that interact with cell surface ligands, δ and Jagged, from neighboring cells. Ligand binding activates a series of proteolytic cleavages and posttranslational modifications, releasing the Notch intracellular domain (NICD) (27). The NICD translocates to the nucleus and associates with the DNA binding protein, RBP-Jκ (28) to form a complex that binds to and activates the promoters of the hairy/enhancer of split (HES) bHLH transcriptional repressors (29). HES1 binds to N-box sequences in promoters to repress the expression of several bHLH transcription factors important for terminal differentiation (30). Readers are referred to any one of a number of reviews of Notch signaling for more details (19, 20).

Notch Signaling Blocks Endocrine Differentiation in the Gastrointestinal Tract

Components of the Notch signaling pathway are present in mouse intestinal epithelium beginning about embryonic d 13.5 and continue through adulthood (22, 31). The first evidence that Notch regulates gastrointestinal endocrine differentiation came from studies on the development of the pancreas in mouse embryos null for either delta 1 or RBP-Jκ. These animals showed arrested pancreatic development with increased NGN3 expression, a marked increase in the number of endocrine cells, and the failure to initiate acinar differentiation. These studies suggested that in the absence of Notch signals, there is precocious endocrine differentiation with resultant depletion of immature epithelial precursor pools (21).

A role for Notch signaling in the regulation of enteroendocrine differentiation was shown in another model of widespread Notch inactivation, mice harboring a deletion of Hes1, a factor downstream of activated Notch. Analysis of Hes1 −/− mice showed a 3- to 7-fold increase in the number of enteroendocrine cells in the stomach and small intestine (22). This was accompanied by elevated expression of the cell surface ligand, delta 1, as well as the proendocrine bHLH proteins Math1, NGN3, and BETA2, suggesting that Hes1 normally inhibits expression of these transcription factors. Also, the NGN3 promoter has several Hes1 binding sites and transfection studies indicate that Hes1 directly inhibits NGN3 expression (32). Taken together, these results indicate that Notch is off in differentiated endocrine cells. These same cells activate Notch signals in neighboring cells thereby blocking endocrine differentiation (Fig. 1).

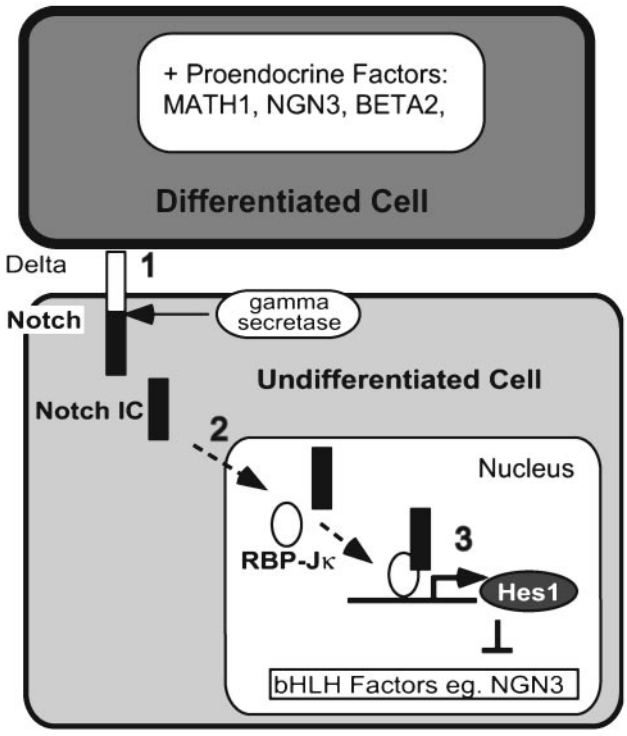

FIG. 1. Lateral inhibition of endocrine differentiation by Notch signaling. 1, Differentiating cells up-regulate the Notch ligand, delta, which binds to the Notch extracellular domain on adjacent cells and activates cleavage of the NICD. 2, NICD migrates to the cell nucleus and interacts with RBP-Jκ to activate transcription of target genes. 3, Downstream target genes include Hes1, a repressor that inhibits proendocrine bHLH transcription factors.

Math1 and NGN3 Are Required for Differentiation of Enteroendocrine Cells

The bHLH gene Math1 is a mammalian homolog of the *Drosophila atonal* gene that plays a pivotal role in neuronal specification as well as functioning as a positive regulator of neuronal differentiation (33, 34). Math1 is expressed in the gastrointestinal tract during development and has been identified in both the immature crypts and villi of the intestinal epithelium but not in the stomach (35). Math1 appears to be important for specification of intestinal secretory lineages as Math1 −/− mice fail to develop three of the four gastrointestinal epithelial cell types—goblet, Paneth, and enteroendocrine cells (35). Cell lineage studies where the β-galactosidase gene was knocked into the Math1 allele, which relies upon the high abundance and long half-life of the β-galactosidase reporter gene, demonstrated Math1 expressing cells become goblet, enteroendocrine, or Paneth cells and suggests that each of these three lineages arise from a Math1-expressing precursor cell (35). The dependence on Math1 expression distinguishes enteroendocrine differentiation from the pancreas where islet differentiation is unaffected in Math1 null mice (Fig. 2).

A second *atonal*-related bHLH protein, NGN3, is required for endocrine cells to differentiate in the gastrointestinal tract (36–38). NGN3 −/− mice fail to develop any endocrine cells in the small intestine. However, in the glandular stomach serotonin cells, enterochromaffin-like cells, and ghrelin-expressing cells differentiate in the absence of NGN3, indicating that these lineages are not dependent upon NGN3 expression (38). This suggests that enteroendocrine determination occurs differently along the gut tube and that other factors are important for endocrine specification in the stomach. NGN3 expression is detected as early as embryonic d 12.5 in the developing murine intestine and is restricted to proliferating, immature cells in the crypts of the adult intestine as well as the glandular stomach (37, 38). NGN3 has not been identified in cells expressing endocrine differentiation markers in both the pancreas and intestine, indicating that it is transiently expressed, switching off before terminal differentiation (39). NGN3 −/− mice express Math1, suggesting that NGN3 is a downstream target of Math1 in the transcription factor cascade controlling endocrine differentiation (37) (Fig. 2).

Although both gain and loss of function studies in transgenic mice suggest that NGN3 expression is essential for much of 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. Expression of β-galactosidase in transgenic mice under control of 6.9-kb NGN3 gene flanking sequence showed transgene expression in some but not all

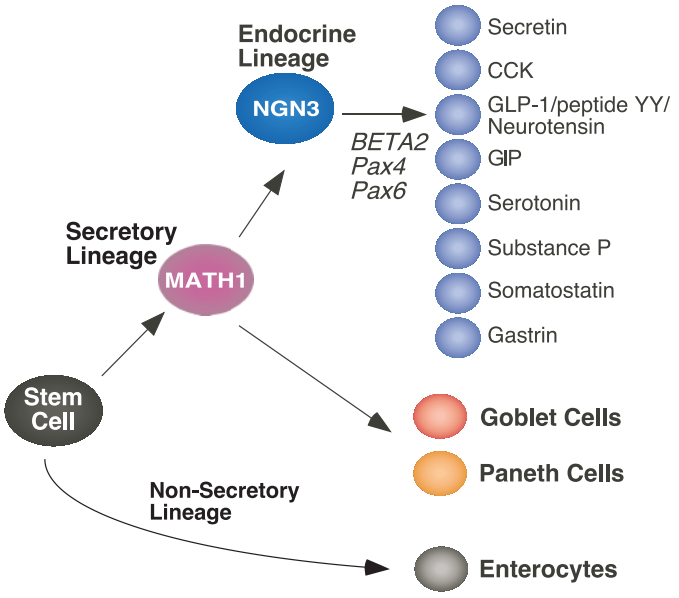

FIG. 2. Schematic overview of enteroendocrine differentiation in the intestinal tract. Stem cells located in the crypts differentiate into all four cell types present in the intestinal epithelium. Math1 expression restricts cells to the secretory lineage and NGN3 restrict cells to the endocrine lineage, whereas the transcription of specific hormones is regulated by several late acting transcription factors such as Pax4, Pax6, and BETA2.

endocrine cells in the intestine and the stomach. A number of enteroendocrine cells showed β-galactosidase colocalized with chromogranin A, a marker of endocrine differentiation, despite the absence of NGN3 expression, which may have been transient in these cells (37). The increased stability of the β-galactosidase reporter beyond the half-life of NGN3 probably accounts for this discrepancy, although the failure to observe β-galactosidase staining in a significant fraction of NGN3+ cells (37) may indicate that the transgene did not completely recapitulate the expression of NGN3, possibly due to the failure to include all regulatory elements important for transgene expression in the stomach and intestine. Although these studies suggested that some NGN3+ cells become endocrine cells, it was unclear whether all gastric and intestinal endocrine cells arise from NGN3+ cells.

Recombination-based cell lineage marking may represent the best approach to determine the eventual cell fate of all NGN3+ cells by marking cells even after NGN3 expression switches off. A more recent study generated a transgenic mouse expressing Cre recombinase under control of 6.5 kb of NGN3 flanking sequence. Crossing this mouse with a Cre indicator strain marked all pancreatic islet cells, indicating that they all arose from NGN3+ cells (40). Similar studies in the intestine will be needed to establish whether all enteroendocrine cells arise from NGN3+ precursors.

Transcription Factors Controlling Terminal Differentiation of Enteroendocrine Cells

Although there is an increasing body of evidence suggesting that most enteroendocrine cells arise from common progenitor cells, relatively little is known about how the approximately 10 different lineages segregate as they differentiate. Whereas some cell lineages are found primarily in the stomach and proximal small intestine (gastrin, ghrelin, GIP, secretin, cholecystokinin), others are predominantly found in the ileum and colon (peptide YY, GLP-1, GLP-2, neurotensin), and still others are found throughout the gastrointestinal tract (somatostatin, serotonin, substance P). The nature of positional cues that direct the rostro-caudal distribution of each cell type have not been characterized thus far.

Because the expression of most gut hormones is restricted to a specific enteroendocrine cell type, elucidation of transcriptional controls regulating expression of a gut hormone gene may provide important insights for understanding how endocrine cells differentiate. Relatively few gut hormone genes have been studied in detail thus far. The sections that follow briefly illustrate the roles of several transcription factors belonging to the bHLH, homeodomain, and paired box homeodomain families in regulating hormone expression (Fig. 2).

The cell type-restricted bHLH transcription factor BETA2, also known as NeuroD1, was originally discovered as a factor important for activating insulin gene transcription and neuronal differentiation (26, 41). BETA2 binds to E box sequences (CANNTG) as part of a heterodimeric complex with ubiquitously expressed bHLH proteins like E12/E47. Several observations suggest that BETA2 is a downstream target of NGN3. BETA2 expression is absent in NGN3 −/− mice and the BETA2 gene is transactivated by NGN3 (37, 42). Subsequent work showed that BETA2 is also an important regulator of secretin and possibly cholecystokinin gene transcription (43). BETA2 null mice fail to develop secretin and cholecystokinin cells in the intestinal tract (44), suggesting that BETA2 is required for the expression of both secretin and cholecystokinin. In addition to its effects on secretin gene transcription, BETA2 induces cell cycle arrest, possibly by increasing expression of p21, an inhibitor of cyclin-dependent kinases (45). In BETA2 null mice, cells expressing the null allele show reduced p21 expression with increased expression of cell proliferation markers, suggesting that BETA2 may function to coordinate expression of secretin with cell cycle arrest and terminal differentiation.

Pdx1, a homeobox protein, regulates insulin and somatostatin gene expression in the endocrine pancreas (46, 47). Most intestinal Pdx1 expression is restricted to the proximal duodenum during fetal development, where it is believed to mediate foregut competence to form pancreas. The number of enteroendocrine cells expressing serotonin, secretin, and cholecystokinin is reduced in this region of the duodenum but not elsewhere in Pdx1 −/− mice (48). Gastrin cells are absent in Pdx1 −/− mice, which suggest that Pdx1 is required for gastrin cell maturation (49). However, Pdx1 has not been shown to be critical for gastrin transcription because not all gastrin cells express Pdx1 (49). It is not clear whether the changes in enteroendocrine cells populations seen in Pdx1 null mice results from direct activation of each gene by Pdx1 or by an indirect mechanism. In the case of the secretin gene enhancer, there are no known functionally important elements resembling a Pdx-1 binding site (50).

Pax4 and Pax6 are paired box homeodomain transcription factors implicated in pancreatic and intestinal endocrine cell fate determination (51, 52). Pax6 is required for normal pro-

glucagon gene expression in both the pancreas and intestine. Deletion of Pax6 eliminates GLP-1- and GLP-2-expressing cells in the distal intestine (52), gastrin and somatostatin cells in the antrum, and GIP cells in the duodenum (51). In addition, reduced numbers of all four pancreatic islet cell types was seen in Pax6 null mice (53), and pancreatic endocrine cells were absent in Pax4 and Pax6 double knockout mice (54). Pax4 null mice lack serotonin and somatostatin cells in the antrum as well as most endocrine cell types in the proximal small intestine (51). The absence of these endocrine cell types in null mice indicates that Pax4 and Pax6 may be required for normal enteroendocrine differentiation.

However, it is not known whether Pax4 and Pax6 directly activate transcription of all of the affected hormones. Transfection experiments indicate that Pax6 binds to regulatory elements in the proglucagon enhancer to increase proglucagon transcription (55, 56). Proglucagon mRNA transcripts were significantly reduced in mice carrying a dominant-negative mutation in Pax6 and GLP-1 and GLP-2 cells were not detected in the intestinal epithelium (52). The presence of peptide YY-expressing cells in Pax6 null mice (Ratineau, C., and A. Leiter, unpublished observations), suggests that the absence of Pax6 does not disrupt the terminal differentiation of L cells in the ileum and colon. *In vitro* observations show that Pax4 and Pax6 may be targets of proendocrine bHLH transcription factors. NGN3 appears to activate Pax4 expression (57, 58), whereas BETA2 may activate PAX6 (59). Therefore, Pax4 and Pax6 are likely downstream effectors of the Math1-NGN3-BETA2 cascade and act to restrict endocrine progenitors to a specific endocrine lineage (Fig. 2).

Future Questions

Many open questions remain regarding how different enteroendocrine cells are specified. Very little is known about the transcriptional and signaling events that direct highly cell type- and region-specific expression of hormones in the gut. Differentiation of intestinal epithelial cells, of which enteroendocrine cells comprise a very minor fraction, is not well characterized, further emphasizing the complexity of the gastrointestinal tract. Although Math1 and NGN3 are important for the global initiation of endocrine differentiation in the intestine, the signals and pathways involved in defining specific endocrine cell types in the proximal intestine *vs.* the colon remain unknown. Relatively little is known about how enteroendocrine cell lineages segregate from their precursor cells as they terminally differentiate within a given region of the gastrointestinal tract. Future work in transgenic mouse models will contribute to identifying new factors as well as understanding the spatial and temporal expression of these factors during endocrine differentiation. Furthermore, the answers to these questions will depend in part on the analysis of how gut hormone gene expression is regulated. For many years, it was believed that the major function of gastrointestinal hormones was to regulate other digestive organs like the stomach, pancreas, and intestine. However, hormones of the gastrointestinal tract have been increasingly implicated in the regulation of a number of physiological processes unrelated to digestive organ function such as appetite regulation via the central nervous system and insulin secretion from pancreatic β cells. The ability to potentially modify different populations of enteroendocrine cells may become an important therapeutic strategy for treating and/or preventing a variety of common human diseases like diabetes and obesity.

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