2012-08-27

Serotonin-Expressing Cells in the Corpus of the Stomach Originate from Bone Marrow: A Master's Thesis

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SEROTONIN-EXPRESSING CELLS IN THE CORPUS OF THE STOMACH ORIGINATE FROM BONE MARROW

A Master's Thesis Presented

By

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Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

AUGUST 27TH, 2012

BIOMEDICAL SCIENCE

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Interdisciplinary Graduate Program

August 27th, 2012

Acknowledgements

This work would not have been possible without the aid and advice of numerous people. Walden Ai of Columbia University provided a BAC for producing the HDC-CFP transgenic mouse, as well as primer sequences. Theresa Keeley of the Linda Samuelson lab at the University of Michigan provided a protocol and help for dissociating mouse stomach epithelial cells. The members of the Anne Rothstein and Ellen Gravallese labs at the University of Massachusetts Medical School provided tissue from bone marrow transplant mice. John Evans III of the Leslie Berg lab at University of Massachusetts Medical School offered suggestions for characterizing mast cells. The Flow Cytometry Core Lab at the University of Massachusetts Medical School provided much help and patience in enriching my low percentage and sometimes only faintly fluorescent cells. Andrew Leiter and the past and present members of his lab offered constant advice and direction.

Abstract

Neurogenin 3 and its downstream target NeuroD are basic helix-loop-helix transcription factors which promote endocrine differentiation in the gastrointestinal tract. However, mice lacking Ngn3 still produce several hormones in the stomach. Lineage tracing mouse models demonstrated that a majority of hormone cells in the corpus region of the stomach did not express Ngn3 or NeuroD during differentiation. Serotonin and histamine cells were entirely NeuroD-independently derived, and serotonin cells were additionally entirely Ngn3-independently derived. In this study, we isolated serotonin and histamine cells from the gastric corpus of transgenic mice expressing the fluorescent marker CFP. Serotonin cells expressed multiple mast cell markers by RT-PCR, and were found to be nearly absent in a mast cell-deficient mouse model. Labeled bone marrow transplant mice showed all serotonin cells derived from bone marrow. Histamine-expressing ECL cells, while lacking NeuroD, did not appear to express granulocyte or mast cell markers by analytical flow cytometry and RT-PCR, and resemble other enteroendocrine cell populations. Mouse gastric corpus serotonin cells, but not antral serotonin cells, are bone marrow-derived mast cells.

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List of Abbreviations

5HT – serotonin (5-hydroxytryptamine)

HDC – histidine decarboxylase

Tph1- tryptophan hydroxylase 1

ECL – enterochromaffin like

CFP – cyan fluorescent protein

eGFP – enhanced green fluorescent protein

eYFP – enhanced yellow fluorescent protein

BAC – bacterial artificial chromosome

Preface

The generation of the Tryptophan Hydroxylase 1-CFP transgenic mouse was done by Nirmal Singh of the Leiter lab. His work included designing primers, performing the recombination steps, and preparing the final construct for the transgenic mouse core facility. These mice were characterized by immunofluorescence by me and Nirmal Singh.

The BAC containing the HDC gene was provided by Walden Ai at Columbia University. He aided in primer design by providing the primer sequences he designed for production of the HDC-GFP mouse, published in Yang et al., 2011.

The GFP-labeled bone marrow transplant mouse stomach tissue was provided by members of the Rothstein and Gravallese labs at the University of Mass Medical School. They performed the irradiation and transplantation, and provided me with tissues from these mice.

INTRODUCTION

The transcription factor Neurogenin 3 (Ngn3) is a basic-helix-loop-helix transcription factor that promotes endocrine differentiation. It was first identified in 1996 in a screen for proteins with high homology to the *Xenopus* Neurogenin protein (Sommer et al., 1996). The proteins characterized in mammals as Neurogenin 1 and Neurogenin 2 were found to have partially overlapping expression patterns in neuronal cells, while Neurogenin 3 is expressed in endocrine cells of the gastrointestinal tract.

Neurogenin 3 promotes differentiation of endocrine cells in the stomach, intestines, and pancreas. Ngn3 is transiently expressed in precursor cells of all three tissues (Schwitzgebel et al., 2000). These precursor cells then give rise to a considerable number of endocrine subtype cells expressing different hormones, which vary by tissue. In the stomach, five subtypes of endocrine cells reside in the lower portion of gastric pits, which are invaginations in the surface of the gastric epithelium (Fig 1). Expression of downstream target genes, including NeuroD, continues terminal differentiation of adult endocrine cells by promoting cell cycle exit and, in some cases, directly binding hormone gene promoters (Mutoh et al., 1998). Each transcription factor is sufficient to produce endocrine differentiation in expression studies, with Ngn3 thought to activate NeuroD (Apelqvist et al., 1999, Grapin-Botton et al., 2001, Gasa et al., 2004, Schwitzgebel et al., 2000). Together, Ngn3 and NeuroD make up a cascade of transcription factors to promote endocrine differentiation.

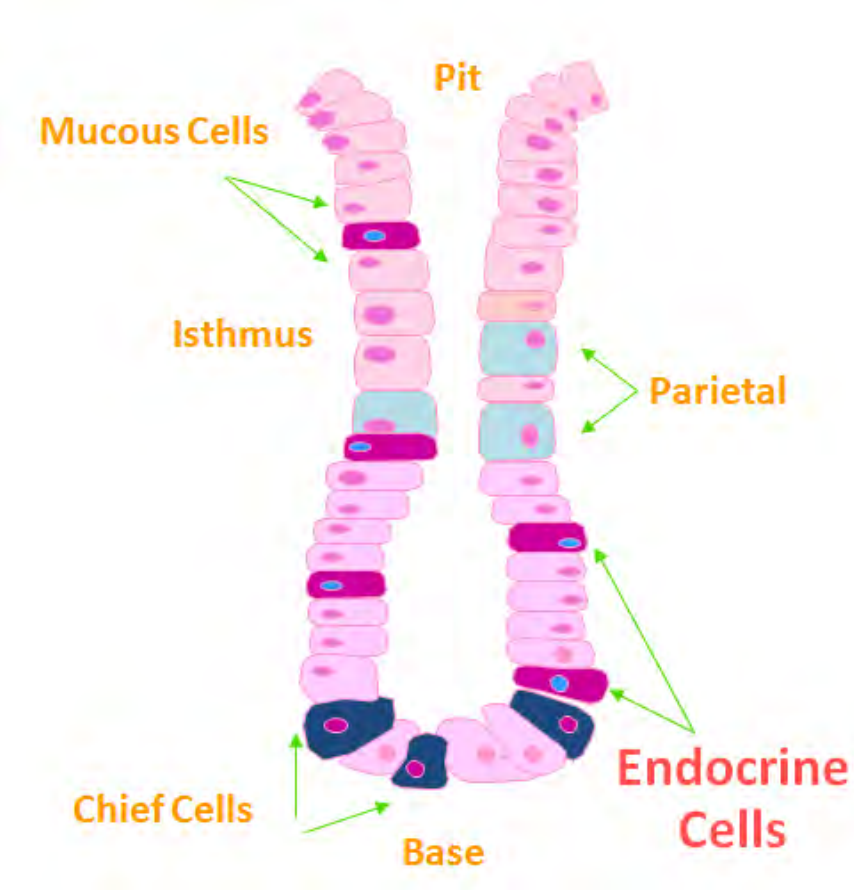

Figure 1: Structure of a Gastric Gland. The gastric gland is an invagination in the gastric mucosa found in the glandular regions (the corpus and antrum) of the mouse stomach. The gland consists primarily of four cell types. Parietal cells secreted H+ to create the acidic conditions of the stomach, while mucus is secreted by mucous neck cells to protect the stomach lining against the acidity. Chief cells are responsible for secreting digestive precursor enzymes, namely pepsinogen and chymosin. Hormone-producing endocrine cells reside near the base of the pits, below the isthmus, the narrowing of the gastric gland.

In 2002, two different studies characterized mice deficient for Neurogenin 3 (Jenny et al., 2002, Lee et al., 2002). Mice born without Ngn3 expression died 1-2 days postnatally, the result of hyperglycemia brought on by the lack of insulin-producing pancreatic beta cells. However, immunohistochemistry performed on the stomach of Ngn3-deficient neonates showed the presence of multiple hormones, as well as expression of the pan-endocrine marker chromogranin A (ChgA) (Jenny et al., 2002). Two hormones, serotonin and ghrelin, were present in the neonatal stomach tissue. A third hormone, histamine, which only arises in the adult stomach, later appeared in isografts of Ngn3-deficient stomach tissue transplanted subcutaneously in nude mice. Hormone expression in Ngn3-deficient mice appeared to be limited to the stomach, as no hormones were detectable in the intestines or pancreas. The implication of this finding was that Ngn3 is not necessary for differentiation of all enteroendocrine cells.

The Ngn3 downstream target NeuroD is additionally important in endocrine differentiation. Like Ngn3-deficient mice, NeuroD-deficient mice die perinatally as a result of hyperglycemia (Naya et al., 1997). However, analysis of NeuroD-deficient mice showed that NeuroD expression was necessary only for the hormones secretin and cholecystokinin, expressed in small intestine. Other hormone populations like pancreatic insulin, though 40% reduced, were still expressed. These mice also show defects in the central nervous system, as NeuroD is important for inducing neuronal differentiation as a target for Ngn1 and Ngn2 (Schwab et al., 2000, Sommer et al., 1996).

The discovery of endocrine cells arising independently of Ngn3 expression led to the development of a model to determine which hormone cells actually express Ngn3 during their differentiation under wild-type conditions. The transient low level expression of Ngn3 makes antibody staining of Ngn3 difficult, as adult cells derived from Ngn3-expressing precursors typically appear Ngn3-negative. To that end, a Ngn3-Cre transgenic mouse was created and bred to a Rosa26-LacZ indicator mouse. The resulting recombination causes cells that expressed Ngn3 at any point during their differentiation to express β-galactosidase (β-gal) (Schonhoff et al., 2004, Soriano, 1999).

Immunohistochemistry performed for different hormones showed that the large majority of hormone-expressing cells in the central part of the stomach, the corpus, did not derive from a Ngn3-expressing precursor. The regions of the mouse stomach are illustrated in Figure 2. Additionally, a small percentage of serotonin cells in the small intestine did not express Ngn3 during differentiation. Pancreatic islet cells were entirely Ngn3-derived. Interestingly, large percentages of Paneth and goblet cells of the small intestine also expressed LacZ. This indicated that Paneth, goblet, and enteroendocrine cells derive from a common secretory precursor cell which can express Ngn3, and that a threshold of Ngn3 expression must be met to achieve endocrine cell differentiation. Similarly, a small number of duct and acinar cells in the pancreas also arise from Ngn3-expressing precursors (Schonhoff et al., 2004).

A follow-up to the Ngn3 lineage tracing study used a second transgenic mouse to determine how many endocrine cells express NeuroD during differentiation. A NeuroD-

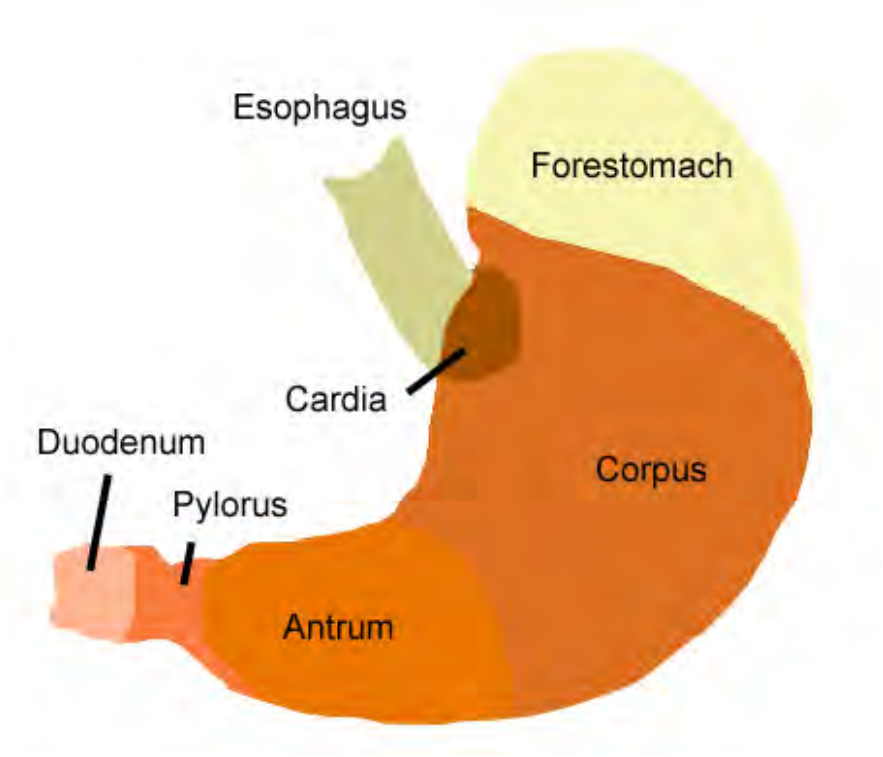

Figure 2: Anatomical Regions of the Mouse Stomach. The regions of the mouse stomach differ from human stomach. Mice have a forestomach, a region of squamous epithelium more similar to esophagus than the rest of the stomach, which constitutes approximately one-third of the stomach area. The corpus (body) and antrum consist of glandular epithelium, which is populated with pits that form the gastric glands.

Cre transgenic mouse was bred to a Rosa26-LacZ indicator mouse, labeling all cells expressing NeuroD with β-gal expression. These mice showed an overall similar pattern to the Ngn3 lineage tracing mouse in derivation, but with distinct differences in a few populations. In the antral stomach, hormone-expressing cells were generally NeuroD-expressing, with the exception of some serotonin and ghrelin cells. In the corpus, however, not all hormone-expressing cells arose from NeuroD during differentiation; many were found to be XGal-negative. In particular, no serotonin cells and no histamine cells appeared to have expressed NeuroD (unpublished observations).

The existence of hormone-expressing cells in the corpus which do not derive from Ngn3-expressing precursors is demonstrated in both the Ngn3-knockout and the lineage tracing mouse. Similarly, hormone-expressing cells that arise in the absence of NeuroD have also been detected in the gastric corpus of lineage tracing mice. These cells must differentiate in the absence of these primary endocrine-promoting transcription factors, Ngn3 and NeuroD. In this study, we sought to identify transcription factors which promote endocrine differentiation in the absence of Ngn3 and NeuroD expression. To this end, we studied those endocrine cells of the stomach which never express Ngn3 or NeuroD during their differentiation. Serotonin-producing cells in the gastric corpus, unlike the other hormone-expressing cells of the corpus, appeared to be a uniformly Ngn3- and NeuroD-independent population. This uniformity, along with its diverse expression pattern and biological functions, made it an excellent candidate for study. Similarly, the histamine cell population was found to derive without any NeuroD expression. The histamine cells served as a parallel target population for this study, which

due to its abundance in the gastric corpus made it well-suited for isolation. These uniform Ngn3- and NeuroD-independent endocrine cells can be isolated from the gastric corpus using fluorescent-labeling transgenic mice which labeled the endocrine cells for enrichment by flow cytometry. These cells can be used to identify transcriptionally highly-expressed gene products which promote endocrine differentiation in the absence of Ngn3 and NeuroD.

The identification of a Ngn3-independent means of endocrine differentiation would have implications for all endocrine cells of the gastrointestinal tract, by piecing together the puzzle of endocrine differentiation. And further elucidation of the differentiation of serotonin cells may shed light on the origin of the carcinoid tumor, a rare neuroendocrine tumor characterized by overabundance of serotonin cells. A cell of origin has not been identified for this cancer, and this apparently distinct serotonin population may provide clues to its origin.

METHODS

BAC Transgene Construction

Two transgenic mice were produced by homologous recombination in *E. coli*. All mouse work was done under the approved IACUC protocol of Dr. Andrew Leiter.

HDC-CFP mouse

The HDC-CFP bacterial artificial chromosome (BAC) transgene was generated from the BAC clone RP23-474H6. A linear fragment of DNA containing the CFP coding region and sequences homologous to the HDC coding region was amplified from a plasmid containing the CFP sequence and a Frt-flanked kanamycin resistance cassette using 65mer primers. (Sense: CCTTCCAGCCTCCTCTGTCTGTCTGCCAGGAGGAGCAATCCAAGGGAGATGG TGAGCAAGGGCGA. Underlined sequence has 48 bases of homology to HDC region immediately upstream of start codon, remaining sequence is 17 bases homologous to start of CFP coding region. Antisense: AGCTCGGTAGTATTCACG GTATTCACGGTATTCACAGGGCTCCATCATCCTCCTTAGTTCCTATT. Underlined sequence is 48 bases of homology to start of HDC coding region, remaining sequence is 17 bases homologous to kanamycin cassette). EL250 cells containing the BAC clone RP23-474H6 and the λ phage red system (generously provided by W. Ai, Columbia University) were made competent and electroporated with the amplified fragment (Cotta-de-Almeida et al., 2003). The fragment was then inserted ahead of the

HDC start codon via homologous recombination. BACs containing the recombined fragment were selected for kanamycin resistance. The kanamycin resistance cassette was excised by transfection with pCP20 plasmid containing Flp recombinase. The construct accuracy was tested by DNA sequencing and field inversion gel electrophoresis. The BAC transgene was purified for pronuclear injection using QIAGEN Large-Construct Kit.

Tph1-CFP mouse

The Tph1-CFP BAC transgene was generated from the BAC clone RP23-28A7. This BAC was obtained from the Children's Hospital Oakland Research Institute (CHORI) BAC library. A linear fragment of DNA containing the CFP coding region and sequences homologous to the Tph1 coding region was amplified from a plasmid containing the CFP sequence and a Frt-flanked kanamycin resistance cassette using 60mer primers. (Sense: CACTCATTTATCTGAACTTTCACACTTCAGATTCACCATGATGGTGAGCAAGG GCGAGGA. Underlined sequence has 40 bases of homology to Tph1 region immediately upstream of start codon, remaining sequence is 20 bases homologous to start of CFP coding region. Antisense: AATGGTCTTTGTTCTCTTTGTTCTCCTTGTTGTCTTCAATTCCTCCTTAGTTCCT ATTCCGA. Underlined sequence is 40 bases of homology to start of Tph1 coding region, remaining sequence is 20 bases homologous to kanamycin cassette). The construct was electroporated into *E. coli* containing BAC RP23-28A7 and the λ phage red system. The fragment was then inserted ahead of the Tph1 start codon via homologous

recombination. BACs containing the recombined fragment were selected for kanamycin resistance. The kanamycin resistance cassette was excised by transfection with pCP20 plasmid containing Flp recombinase. The construct accuracy was tested by DNA sequencing and field inversion gel electrophoresis. The BAC transgene was purified for pronuclear injection using QIAGEN Large-Construct Kit.

Production of Transgenic Mice

The BAC transgenes were diluted to a concentration of 100 ng/ul in Tris-HCl buffer and provided to the University of Massachusetts Transgenic Mouse Core for pronuclear injection into C57BL/6 x SJL F_2 hybrid mouse embryos. Six founder lines were produced for HDC-CFP. DNA extracted from ear punches was used for genotyping using primers encompassing the HDC promoter and the CFP coding sequence. Sense: TTCCAGCCTCCTCTGTCTGT. Antisense: GCGGATCTTGAGGTCACCTTGATGCC. Four founder lines were produced for Tph1-CFP. Tph1-CFP mice were genotyped using primers including the Tph1 promoter and the CFP coding sequence. Sense: TTGTCTCATGAGCGGATACATATT. Antisense: TGTCTTCAATTCCTCCTTAGTTCC.

Stomach Cell Isolation

Gastric corpus epithelial cells were isolated using a mix of mechanical and enzymatic dissociation. The mouse stomach was resected, and the forestomach and antral portions were removed from the stomach and discarded. The remaining band of gastric corpus

tissue was inverted on a weighted PCR tube. The tube was placed in 8 ml of protease media in a 50 ml conical tube. Media was made from 100 ml Basal Medium Eagle (Gibco), 100 µg bovine serum albumin (Sigma-Aldrich), 50 µg pronase (Sigma-Aldrich), and 1.4 mL 1M HEPES buffer. The conical tube was incubated for 30 minutes on a shaker at 100 rpm at 37°C. The media was then replaced with 8 ml fresh media and the 30 minute incubation was repeated. The tissue was then removed from incubation and vigorously shaken with forceps into 8 ml fresh protease media for five minutes to dissociate the epithelial cells. For downstream cell sorting experiments, cells from multiple stomachs were pooled at this stage in the same 8 ml media. The dissociated cells were then incubated for an additional 30 minutes on a 100 rpm shaker at 37°C. The cells were pipetted for five minutes using a 200 µl tip nested on a 10 ml tip to separate the cells into a suspension of single cells. The cell suspension was passed through a 40 µm filter into a new 50 ml conical tube. The filtered cells were centrifuged for 5 minutes at 1500 rpm. The supernatant was carefully decanted and the cell pellet was resuspended in 1 ml cold 1x HBSS. Gastric epithelial cells tend to clump, and the cells sometimes necessitated further pipetting to maintain a single cells suspension, depending on the concentration of cells. Addition of DNAse after the cell dissociation step was also occasionally used for highly sticky cell suspensions, to digest the free DNA released from dead cells that can cause clumping. For Tph1-CFP cell isolation, nine mice were pooled, and 30,000 cells were collected. For Ngn3-Cre;Rosa26-eYFP cell isolation, seven mice were pooled, and 30,000 cells were collected. For HDC-CFP, four mice were pooled, and

35,000 cells were collected. For each FACS experiment, a single CD1 mouse was used as a negative control.

Tissue Preparation

Mouse tissues were fixed in 4% paraformaldehyde for 3 hours. They were then washed with PBS and placed in 30% sucrose solution overnight at 4°C. The tissues were then embedded in OCT compound (Sakura) and frozen. The frozen tissue blocks were cut into 5 micron sections and mounted on slides.

Immunofluorescence

Immunofluorescence was performed in frozen tissue sections using the following dilutions: rabbit anti-5HT at 1:20,000 (Immunostar), guinea pig anti-HDC at 1:300 (EuroProxima), and rabbit anti-Chromogranin A at 1:1000 (Immunostar). Primary antibodies were detected with the following secondary antibodies: donkey anti-rabbit A594 at 1:2000 (Alexa), and goat anti-guinea pig A594 at 1:2000 (Alexa). CFP and GFP were detected using rabbit anti-GFP A488 conjugated antibody at 1:1000 (Alexa).

Flow Cytometry

Isolated corpus cells were pooled into 1 ml 1x HBSS buffer. Cells were collected on a FACSAria Cell Sorter by the University of Massachusetts Medical School Flow Cytometry Core Lab.

Analytical flow cytometry cell samples were centrifuged at 800 rpm, decanted, and incubated for 10 minutes with selected fluorescently-conjugated antibodies: rat anti-CD45-APC (eBioscience), rat anti-c-Kit-PE (eBioscience), rat anti-Gr-1-FITC (eBioscience), and rat anti-CD11b-PE-Cy7 (eBioscience). Analytical flow cytometry was performed on a BD LSR II flow cytometer.

Bone Marrow Transplant Mice

Stomach tissue from mice that underwent bone marrow transplant was generously provided by the labs of Anne Rothstein and Ellen Gravallese, UMass Medical School. 8-10 weeks old Balb/C recipient mice were lethally irradiated with 850 rads. Four hours after irradiation, the mice were injected with 10 million bone marrow cells by tail vein injection. The bone marrow donor mice were age-matched and sex-matched MHC I-GFP mice. The irradiated mice were fed with acidic water for four weeks, and then switched to regular water. The mice were sacrificed after 12 weeks, and the stomach tissues fixed by the method described under Tissue Preparation. Bone marrow transplantation experiments were performed under the approved IACUC protocol of Dr. Ann Rothstein.

RT-PCR

RNA was produced from FACS-sorted cells by Trizol extraction. Cells collected by flow cytometry into cold 1x HBSS buffer were centrifuged and decanted. Pellets received 0.5 ml Trizol (Invitrogen). Then chloroform was added and the lysed cells were centrifuged to separate the RNA into the aqueous phase. RNA was precipitated with isopropyl

alcohol and washed with ethanol, then dissolved in 15 µl of nuclease-free water. cDNA was produced using this RNA with iScript cDNA synthesis kit (Bio-Rad). To analyze expression of transcripts, primers were designed against several targets. Samples were amplified for 33 cycles. Primer sequences, temperatures, and product sizes are listed in Table 1.

Gene	Primer Sequence		Annealing Temp (°C)	Product Size (bp)
Tph1	CCTTGGAGCTTCAGAGGAGA	Forward	55°	91
	CAGCTGTCCATCTTGTTTGC	Reverse		
HDC	TAAGAGGCACCTTGGTTTGG	Forward	58°	200
	CTTGGATGAGGTGCCAATCT	Reverse		
NeuroD	AAGGTTTGTCCCAGCCCACTAC	Forward	55°	351
	CGGATGGTTCGTGTTTGAAAGAG	Reverse		
MCPT1	AGAAGCTCACCAAGGCCTCA	Forward	54°	971
	GCAAGAGGTTAGGTCTTTATTG	Reverse		
CD34	TCTCTGAGATGGCTGGTGTG	Forward	55°	201
	GGGTAGCTCTCTGCCTGATG	Reverse		
β-actin	CGTGGGCCGCCCTAGGCACCA	Forward	57°	242
	TTGGCTTAGGGTTCAGGGGGG	Reverse		
c-Kit	AAGACCGAGGCTGAGAAGGAAC	Forward	55°	404
	CAGAGCAAGTATGTTGGCTAAGAGG	Reverse		
18S	GTGGAGCGATTTGTCTGGTT	Forward	55°	200
	CGCTGAGCCAGTCAGTGTAG	Reverse		
eYFP	GGAGCGGGAGAAATGGATATG	Forward	56°	500
	AAAGTCGCTCTGAGTTGTTAT	Reverse		
ChgA	ACAACAGGATGGCTTTGAGG	Forward	56°	201
	GCACTCAGGCCTCTCTCACT	Reverse		

Table 1: RT-PCR Primer Design and Conditions.

RESULTS

Production and Characterization of a Tph1-CFP Transgenic Mouse

Serotonin-producing cells in the gastrointestinal tract are a relatively rare population, and represent very small percentages of the total epithelium (Roth et al., 1990). Cell counts in tissue and flow cytometry suggest they comprised 0.7% of the corpus epithelium. In order to enrich the serotonin-producing cells for study, we created a transgenic mouse to express cyan fluorescent protein (CFP) under the control of the gene tryptophan hydroxylase-1 (Tph1), the rate-limiting enzyme in serotonin synthesis in the gastrointestinal tract (a second enzyme, tryptophan hydroxylase 2, produces neuronal serotonin) (Walther et al., 2003, Gutknecht et al., 2008).

We used a recombineering approach to produce a transgenic mouse which labels serotonin cells with a CFP fluorescent marker. The coding sequence for CFP was inserted downstream of the Tph1 promoter in a bacterial artificial chromosome (BAC) containing the Tph1 gene (Fig 3). To ensure that the inserted sequence faithfully recapitulated Tph1 gene expression, we performed immunofluorescence staining with serotonin antibody. All serotonin-expressing cells were CFP-expressing, and all CFP-expressing cells were serotonin-expressing, demonstrating the proper expression of the transgene. This was observed in multiple serotonin-expressing tissues of the gastrointestinal tract: small intestine, antral stomach, and the stomach body (Fig 4).

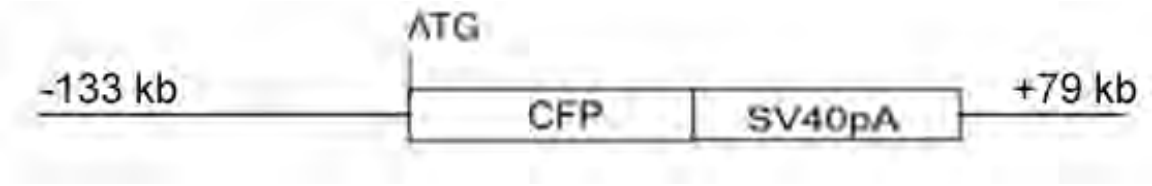

Figure 3. **Generation of a Tryptophan Hydroxylase 1-Cyan Fluorescent Protein (Tph1-CFP) Transgenic Mouse**. Structure diagram of the Tph-CFP BAC construct. The CFP coding sequence was inserted via homologous recombination into a 212-kilobase BAC downstream of the Tph1 promoter. The resulting BAC construct was used to produce a transgenic mouse via pronuclear injection.

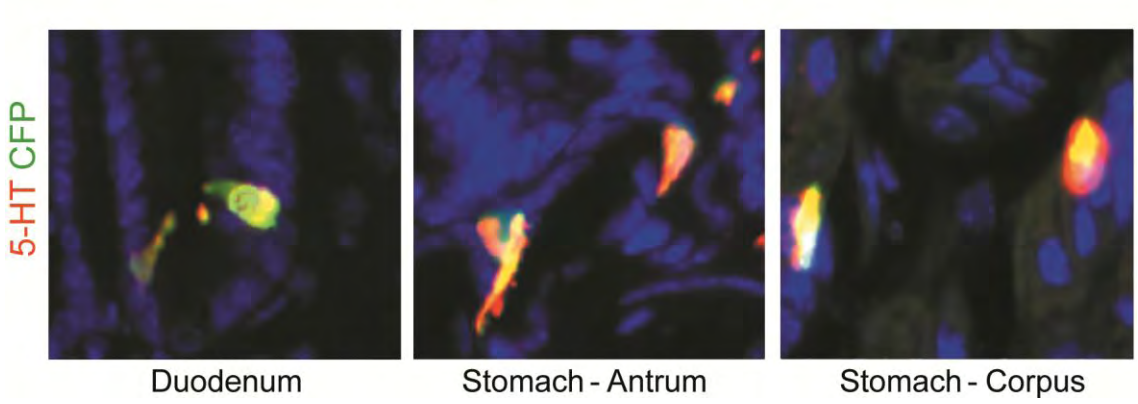

Figure 4. **Tph1-CFP Transgene Recapitulates Serotonin Synthesis Expression Pattern.** Immunofluorscence staining in Tph1-CFP mouse tissue sections show that CFP-expressing cells colocalize with anti-5-HT antibody in duodenum, and the antral and corpus regions of the stomach, of Tph1-CFP mice. Tissue sections were co-stained with serotonin cell marker 5-HT (red) and CFP (green). Colocalization appears in yellow.

Isolation of Serotonin Cells and Ngn3-Derived Cells from Gastric Corpus

Having a model to study corpus serotonin cells, a second cell population was needed for comparison of relative expression levels. To compare the relative levels of selected transcripts of the serotonin cells, we used a previously-described Ngn3-Cre transgenic mouse model to isolate Ngn3-derived endocrine cells from the corpus (Schonhoff et al., 2004). A transgenic mouse expressing Cre in the Ngn3 coding region was bred to a Rosa26-eYFP loxP-flanked indicator mouse, producing a mouse in which all cells that express Ngn3 during their differentiation are labeled with eYFP (Srinivas et al., 2001). To confirm the Ngn3-independence of corpus serotonin cells, immunofluorescence was used to detect serotonin in stomach tissue from this mouse. Using Ngn3-Cre;Rosa26-eYFP mice, we see that 100% of serotonin cells in the corpus arise independently of Ngn3 (Fig 5). At the same time, the eYFP indicator mouse shows serotonin cells in the antrum are 100% derived from Ngn3-expressing precursors. Interestingly, serotonin cells in the corpus tend to reside in the upper half of the gastric glands, unlike the enteroendocrine cells which reside in the base of the glands.

Since the serotonin cell population in the corpus differentiates entirely without Ngn3 expression, the two populations do not overlap and can be sorted by flow cytometry on the basis of single color expression of either CFP or eYFP. Corpus epithelial cells from several mice of each genotype were pooled and sorted by flow cytometry (Fig 6). For each sort, corpus cells from a single wild-type CD1 mouse were

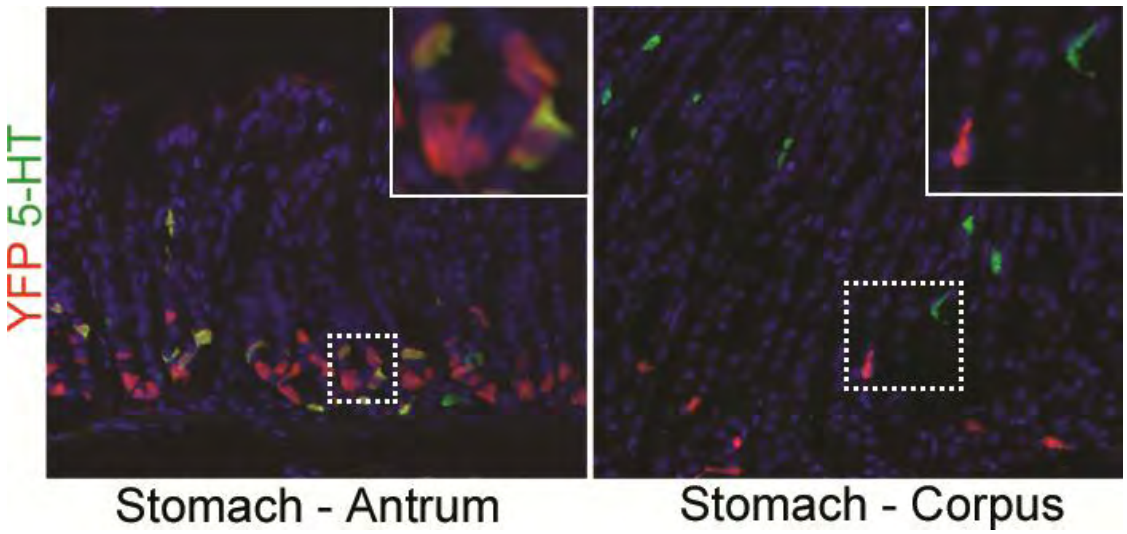

Figure 5. **Identification of Serotonin Cells Arising Independently of Ngn3.** Immunofluorescence using 5HT antibody (green) in Ngn3-Cre;Rosa26-eYFP (red) stomach tissue sections. Nearly all 5HT cells in antral stomach derive from Ngn3-expressing precursors as seen by their colocalization with eYFP (colocalized cells in yellow). In the stomach corpus, serotonin cells do not colocalize with eYFP, and do not arise from Ngn3-expressing precursors.

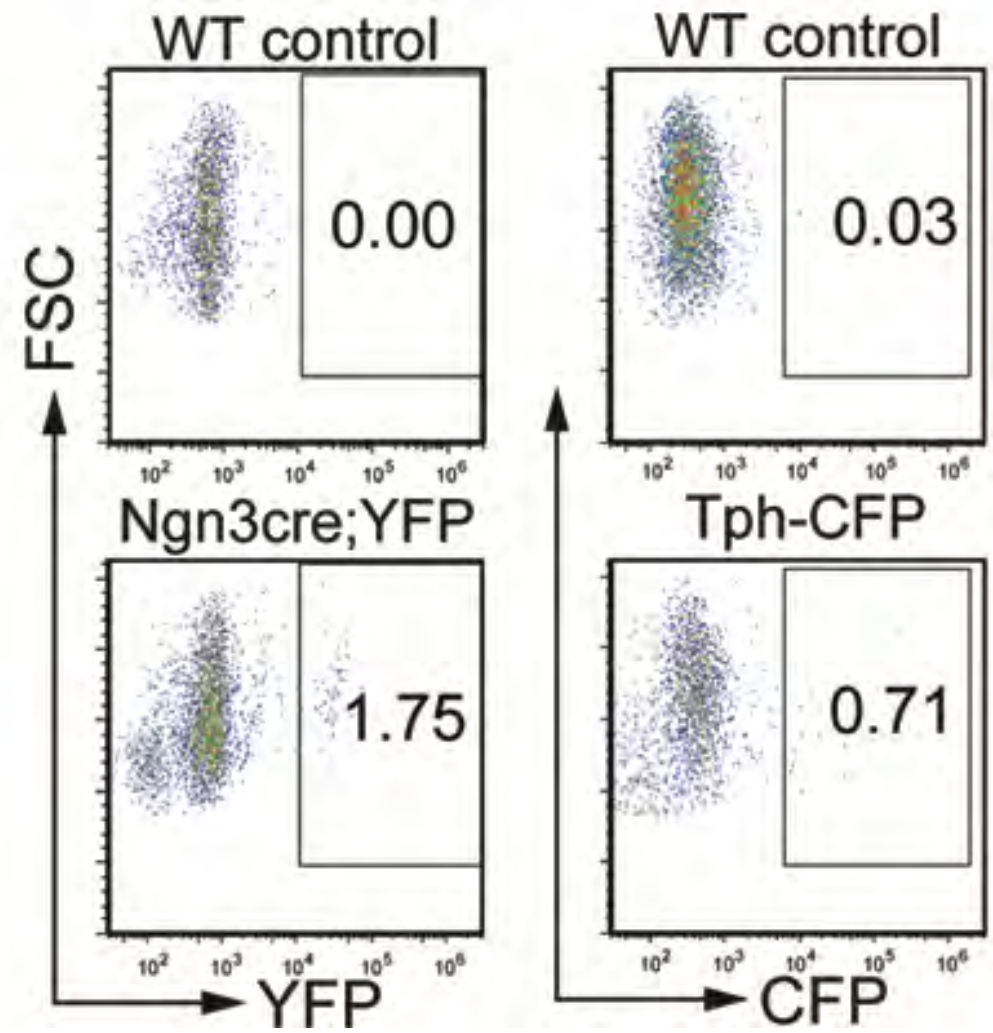

Figure 6. **Tph1-CFP and Ngn3-Cre;Rosa26-eYFP Represent Small Subpopulations of Epithelial Cells in the Corpus**. Flow cytometry analysis shows fluorescently-labeled gastric corpus epithelial cells are small percentages of the total cells isolated. Stomach epithelial cells are isolated from corpus of Tph1-CFP and Ngn3-Cre;Rosa26-eYFP transgenic mice, and sorted on the basis of endogenous fluorescence. CFP and eYFP fluorescence were not detected in wild-type CD1 mice corpus cells used as controls.

used as a negative control. Results show that serotonin cells comprised 0.7% of the total gastric corpus epithelium of Tph1-CFP mice, whereas endocrine cells arising from Ngn3 represented 1.8% of the total gastric corpus epithelium of Ngn3-Cre;Rosa26-eYFP mice. 30,000 CFP+ cells were collected from 9 pooled Tph1-CFP mice, and 30,000 eYFP+ cells were collected from 7 pooled Ngn3-Cre;Rosa26-eYFP mice.

Characterization of Isolated Corpus Serotonin Cells by RT-PCR

To study the profile of these serotonin-producing cells, we isolated the RNA from both the corpus serotonin cells and corpus cells arising from Ngn3-expressing precursors, which was then reverse transcribed to produce cDNA libraries. Candidate gene products for analysis were selected on the basis of several studies. Most crucially, a parallel study in the Leiter lab suggested that several mast cell markers were highly expressed in gut serotonin cells (Leiter, unpublished data). A long history of rodent mast cell studies shows serotonin production in mast cells (Ringvall et al., 2008). Additionally, a recent study showed the majority of histamine-producing cells in mouse bone marrow are related to the granulocyte lineage (Yang et al., 2011). As seen in Figure 7, the serotonin cells do not express NeuroD, consistent with previous results with NeuroD-Cre lineage tracing mice showing no NeuroD expression in corpus serotonin cells. Tph1 expression is absent in the Ngn3-derived cells, confirming that the corpus does not produce Ngn3-derived serotonin cells. The serotonin cells expressed the mast cell markers Mast Cell Protease 1 (MCPT1), c-Kit, and CD34, whereas Ngn3-derived endocrine cells did not.

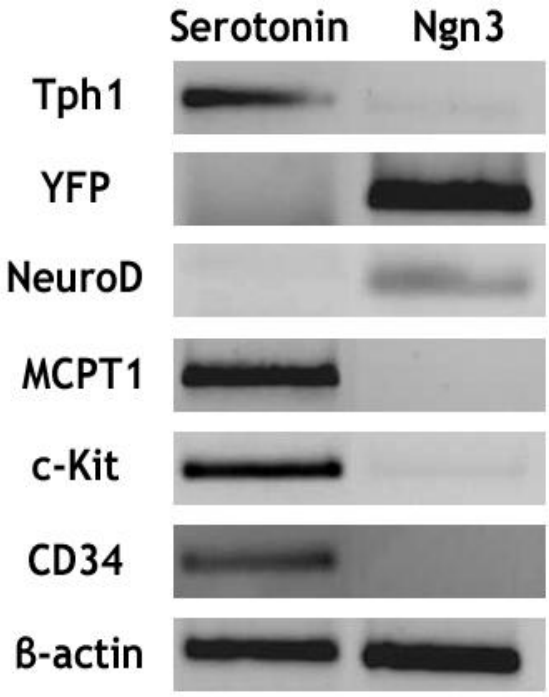

Figure 7. **Serotonin Cells Isolated from Corpus Express Mast Cell Markers by RT-PCR**. CFP+ cells were isolated from the gastric corpus of pooled Tph1-CFP mice and eYFP+ cells were isolated from the gastric corpus of pooled Ngn3-Cre;Rosa26-eYFP mice, and the cells were collected by flow cytometry. RNA was isolated from the collected cells and used to produce cDNA to measure relative expression of selected transcripts. The serotonin (CFP+) cells express the serotonin-producing enzyme Tph1, but no NeuroD. NeuroD is present in the Ngn3-derived endocrine cells (YFP+). c-Kit, CD34 and mast cell protease 1 (MCPT1), all gene products found in adult mast cells, are expressed in the Tph1-CFP cell population, but not in the Ngn3-Cre;Rosa26-eYFP cell population.

In summary, serotonin cells of the corpus do not arise from Ngn3- or NeuroD-expressing precursors, and express multiple mast cell markers.

Serotonin Cells in the Corpus Are Nearly Absent in Mast Cell-Deficient Mice

To determine if corpus serotonin cells are in fact mast cells, we obtained a mast cell-deficient mouse from Jackson Labs. The c-Kit^{Wsh} mouse has a 2.8 Mb inversion in the proximal region of the c-Kit locus (Nagle et al., 1995). Heterozygotes with this inversion are characterized by a white fur sash (Wsh) around its middle. Mice homozygous for this mutation are deficient in both mast cells and melanocytes, as these cell types do not appear to have detectable c-Kit and are not able to differentiate (Duttlinger et al., 1993, Tono et al., 1992). However, hematopoeisis, which is likewise dependent on c-Kit function, occurs normally; c-Kit has normal expression in the rest of the bone marrow cells, and the mice are viable. We examined tissue from three c-Kit$^{Wsh/Wsh}$ mice and found that the serotonin cells are all but absent from the corpus (Fig 8). Total number of cells were counted using DAPI staining in frozen sections, and the number of hormone-expressing cells were counted using anti-hormone antibodies. The hormone cells were taken as a percentage of the total cells to determine change in the percentage of hormone cells between wild-type and c-Kit$^{Wsh/Wsh}$ mice. Each hormone was counted in tissues from three separate mice, and then the mean was calculated. In wild type mice, serotonin cells represented 0.7% of the corpus epithelium, whereas serotonin cells in the corpus of c-Kit$^{Wsh/Wsh}$ mice represented only 0.03% of the gastric corpus

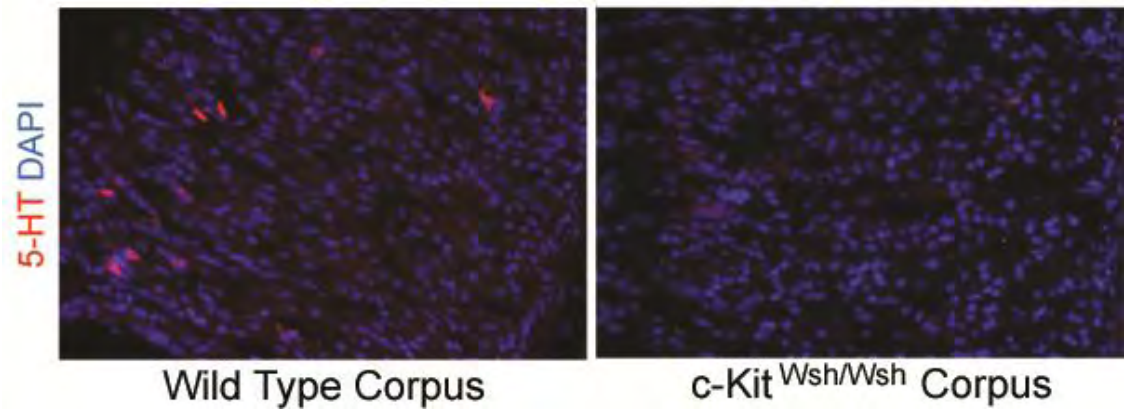

Figure 8. **Serotonin Expression in the Gastric Corpus of Wild-Type and Mast Cell-Deficient Mice**. Serotonin cells in the mouse corpus region are absent in mast cell-deficient mice. Immunofluorescence staining for serotonin in wild type and c-Kit$^{Wsh/Wsh}$ corpus shows serotonin cells are detected in wild type mice, but are only very rarely detected in c-Kit$^{Wsh/Wsh}$ mice.

epithelium. A given tissue section containing the length of the gastric corpus from the mast cell-deficient mouse typically only contained two or three detectable serotonin cells. These serotonin cells are possibly epithelium-derived endocrine cells, or rare mast cells of the sort that have been observed previously in this mouse genotype (Yamazaki et al., 1994).

In contract to the near absence of 5HT cells, ghrelin and somatotstatin expression in cKit$^{Wsh/Wsh}$ mice remains the same (Fig 9). Ghrelin cells represented 1.1±0.22% of the corpus epithelium in wild type, and 1.2±0.15% in c-Kit$^{Wsh/Wsh}$ mice. Somatostatin cells represented 0.5%±0.28% of the corpus epithelium in wild type, and 0.7±0.10% in c-Kit$^{Wsh/Wsh}$ mice. The reduction in serotonin cells is limited to the corpus region. The serotonin population of the antral stomach of wild-type mice and c-Kit$^{Wsh/Wsh}$ mice was unaffected by absence of c-Kit, 1.7±0.01% and 1.7±0.32% of the antral epithelium, respectively. To examine histamine-producing ECL cells, we stained for histidine decarboxylase (HDC), the rate limiting enzyme in histamine biosynthesis. HDC was detected in cKit$^{Wsh/Wsh}$ corpus, demonstrating that these cells are not absent in mast cell-deficient mice. These cells appear unlikely to be mast cells.

Serotonin Cells in the Corpus Are Bone Marrow-Derived

Since the corpus serotonin cells are absent in a mast cell-deficient mouse model, we set out to determine if serotonin cells arise from the bone marrow by using a bone marrow-labeling mouse model. Recipient mice were lethally irradiated to ablate endogenous bone marrow cells, and then given tail vein injections of 10 million donor

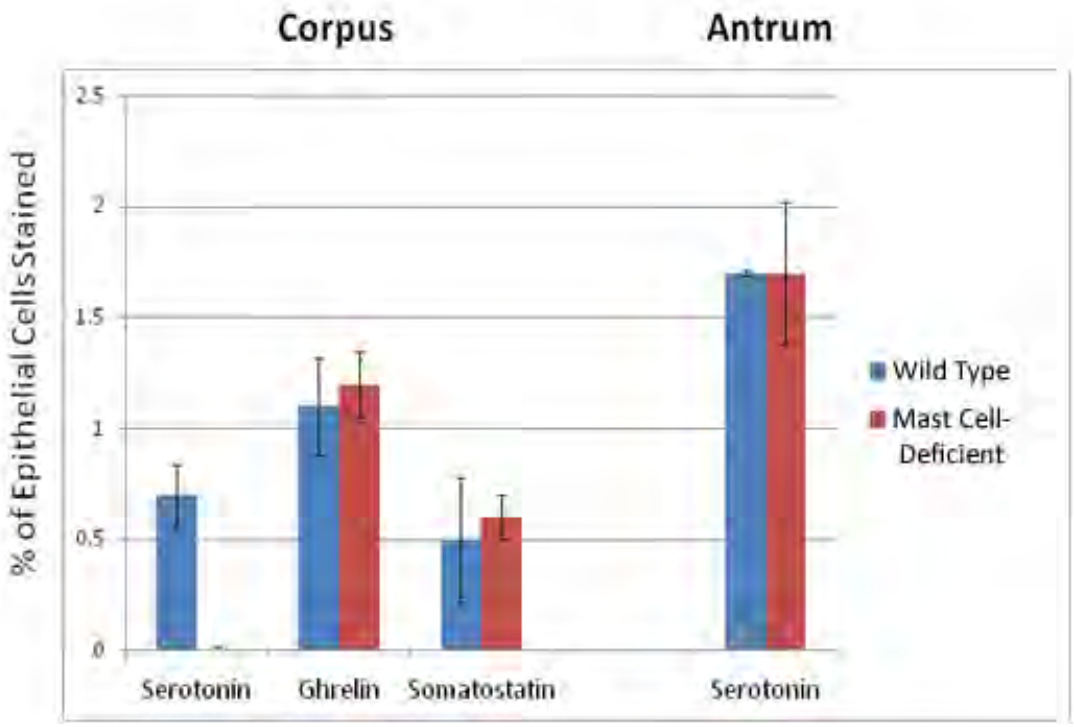

Figure 9. **Hormone Expression in the Stomach of Wild-Type and Mast Cell-Deficient Mice.** Cell counts of hormone-expressing cells in wild type and mast cell-deficient mice were performed in frozen tissue sections. Total cell numbers were determined by counting DAPI-stained cells. Hormone expressing cells were counted using anti-hormone antibodies. Mean percentage of total was calculated for each hormone by averaging counts from three different mice. Serotonin cells are found very rarely in the gastric corpus of mast cell-deficient mice, but other hormone populations appear unaffected by the mutation in the c-Kit promter. Antral serotonin cells, which unlike the corpus serotonin cells arise from Ngn3-expressing precursors, are also expressed at wild-type levels in mast cell-deficient mice.

bone marrow cells from transgenic Balb/C mice expressing GFP under control of the MHC I gene. These irradiated mice were then allowed to recover for twelve weeks for the bone marrow to engraft, with all donor-derived bone marrow cells expressing GFP. In these GFP-labeled bone marrow mice, nearly all serotonin cells in the corpus expressed GFP, revealing that in the transplant model, serotonin cells in the corpus are bone marrow-derived (Fig 10).

The bone marrow origin of hormone-expressing cells in the stomach appears unique to serotonin cells. Ghrelin, somatostatin, and histamine do not colocalize with GFP, which correlates with their unchanged levels in the c-Kit$^{Wsh/Wsh}$ mouse.

Production and Characterization of a HDC-CFP Transgenic Mouse

Histamine cells of the corpus, like serotonin cells, are a NeuroD-independent population of hormone-expressing cells. However, earlier lineage tracing experiments suggest that 30% of histamine cells arise from Ngn3-expressing precursors. To study the histamine cells of the corpus, we used recombineering to produce a transgenic mouse expressing CFP under control of the histidine decarboxylase (HDC) gene, the rate-limiting enzyme in histamine synthesis. We obtained a 213 kb BAC containing the HDC gene, with 113 kb upstream and 75 kb downstream of the HDC gene. 65-base primers with homology to the region upstream of the HDC coding region were used to amplify a construct containing the CFP coding sequence. This construct was then inserted downstream of the HDC promoter by homologous recombination. Immunofluorescence with HDC antibody in gastric tissue showed that HDC expression completely colocalizes

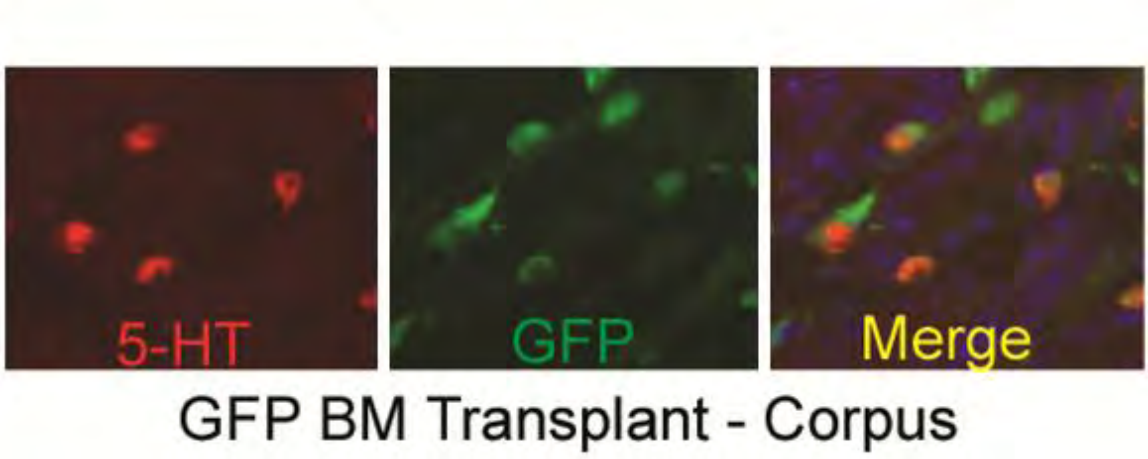

Figure 10. **Serotonin Cells in the Gastric Corpus Are Bone Marrow-Derived.** Immunofluorescence staining of gastric corpus from GFP bone marrow transplant mice shows that most serotonin cells colocalize with GFP, indicating that gastric corpus serotonin cells arise from bone marrow. At the same time, not all GFP+ bone marrow cells express serotonin.

with CFP, demonstrating that the HDC-CFP transgene faithfully recapitulates the HDC expression pattern with the fluorescent marker. Additionally, HDC-CFP cells all express the pan-endocrine marker chromogranin A (ChgA) (Fig 11). This mouse provided a means of studying the histamine cells of the corpus using flow cytometry.

HDC-CFP Cells from Corpus Lack Bone Marrow Surface Markers by Analytical Flow Cytometry

We selected cell markers with which to characterize the histamine cells of the corpus. Like serotonin cells in corpus, the histamine cells do not express NeuroD during differentiation. They also derive largely independently of Ngn3 expression. RT-PCR data generated from corpus serotonin cells suggest that that population is in fact mast cells, derived from the bone marrow. In light of the lineage tracing and RT-PCR data, we set to determine whether histamine cells were likewise bone marrow-derived. We selected a panel of cell surface markers to characterize corpus histamine cells using analytical flow cytometry. Furthermore, a recent study indicates that a majority of HDC-expressing cells in bone marrow are CD11b+/Gr-1+ granulocytes, and that mast cells are not the primary producer of histamine in bone marrow-derived cells (Yang et al., 2011). In light of this information, we included these two granulocyte markers in our panel.

We isolated cells from the gastric corpus epithelium of two HDC-CFP mice, and of a wild-type CD1 mouse control, and incubated with fluorescently-tagged antibodies against the selected panel of cell surface markers (Fig 12). HDC-CFP cells accounted for

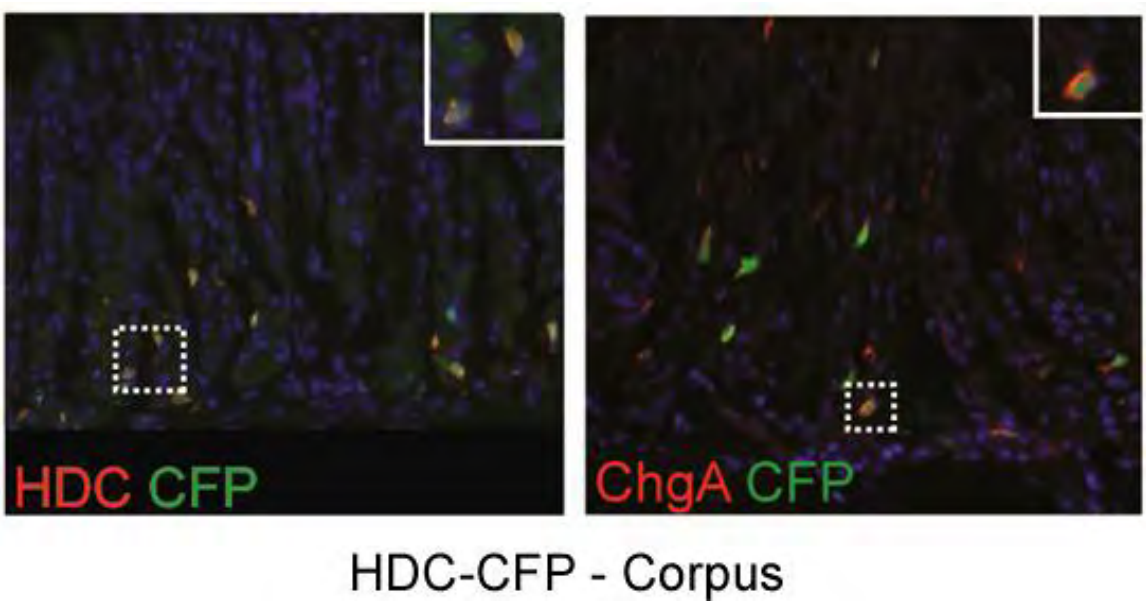

Figure 11. **Histidine Decarboxylase (HDC) Transgene Recapitulates HDC Expression Pattern in Stomach of HDC-CFP Mouse.** Immunofluorescence staining of the gastric corpus of HDC-CFP mice with HDC and ChgA. Both HDC and ChgA colocalize with CFP expression.

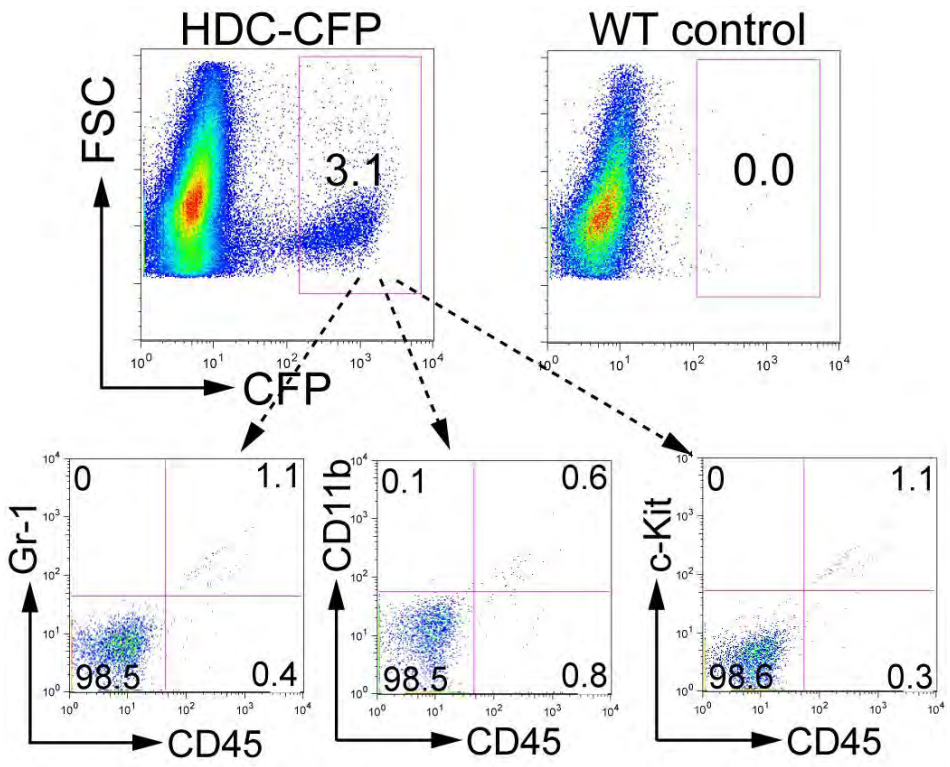

Figure 12. **HDC-CFP Cells from Gastric Corpus Lack Granulocyte and Mast Cell Surface Markers**. FACS analysis of cell surface markers CD45, CD11b, Gr-1 and c-Kit in CFP+ cells isolated from HDC-CFP stomach corpus epithelium. These cell surface markers have negligible amounts of expression in CFP+, suggesting that histamine cells of the gastric corpus are unlikely to be mast cells or granulocytes.

3.1% of the stomach epithelial cells isolated from the corpus. These cells were examined with the granulocyte markers Gr-1 and CD11b, the common leukocyte marker CD45 which is expressed in several adult hematopoetic cells including adult mast cells, and c-Kit, which is expressed in hematopoetic progenitor cells and adult mast cells. Only a tiny fraction of HDC-CFP cells expressed any of the selected markers. CFP+ cells positive for CD45 and Gr-1 were 1.1%, CD45 and CD11b were 0.6%, and CD45 and c-Kit were 1.1%. Flow cytometry suggests that corpus histamine cells are neither mast cells, nor histamine-producing granulocytes. As for the negligible percentages of HDC-CFP cells which did express these markers, it is quite possible that the stomach epithelial cell isolation procedure included a small measure of bone marrow cells from nearby blood vessels, which could account for the low levels of bone marrow marker expression on HDC-CFP cells.

Characterization of HDC-CFP Cells from Corpus by RT-PCR

In addition to using cell surface markers, we measured relative expression of candidate gene products in HDC-CFP cells by RT-PCR. We prepared suspensions of cells from the gastric corpus of HDC-CFP mice and collected CFP-expressing cells by flow cytometry. Four HDC-CFP mice were pooled, and 35,000 CFP+ cells were collected. The collected cells were used for RNA extraction and production of cDNA. For comparison, the HDC-CFP cDNA was compared to cDNA prepared from STC-1 cells, an intestinal endocrine tumor cell line (Rindi et al., 1990).

HDC-CFP cells expressed HDC transcript, reaffirming the identity of the cells isolated (Fig 13). They expressed chromogranin A, consistent with results seen by immunohistochemistry (Fig 11). Furthermore, the HDC-CFP cells lack NeuroD expression, which confirms previous results seen in lineage tracing with NeuroD-Cre mice. The expression of the panendocrine marker ChgA, along with the lack of bone marrow markers by analytical flow cytometry, suggests that HDC-CFP cells in the corpus are enteroendocrine cells arising from the gastric epithelium.

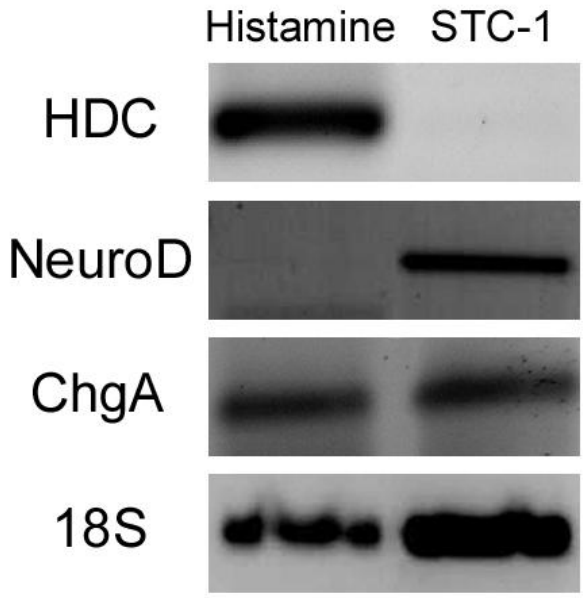

Figure 13. **Histamine Cells Isolated from Corpus Express an Endocrine Marker by RT-PCR**. Histamine (CFP+) cells isolated from gastric corpus of HDC-CFP mice were collected by flow cytometry. RNA was isolated and used to produce cDNA for RT-PCR analysis. For comparison, RNA was isolated and reverse transcribed from cell cultures of the endocrine tumor cell line STC-1. Histamine cells express the enzyme HDC, as well as the endocrine marker ChgA. However, the cells lack NeuroD expression.

DISCUSSION

Neurogenin 3 and NeuroD were both thought to be necessary and sufficient for endocrine differentiation in the gastrointestinal tract. Neurogenin 3 and NeuroD are both sufficient to induce endocrine differentiation in expression studies, and are crucial to the differentiation of endocrine cells in mouse models (Apelqvist et al., 1999, Gasa et al., 2004, Grapin-Botton et al., 2001, Schwitzgebel et al., 2000). However, Ngn3-deficient and NeuroD-deficient mice both produce enteroendocrine cells, suggesting the existence of Ngn3- and NeuroD-independent enteroendocrine cells in the stomach (Jenny et al., 2002, Lee et al., 2002, Mutoh et al., 1998). Lineage tracing mouse models then demonstrated that Neurogenin 3 expression did not lead to downstream NeuroD expression in the case of histamine cells. But these studies also demonstrate that Ngn3-independent hormone expressing cells are not all the same. Histamine cells, while NeuroD-independent, at least partially derive from Ngn3-expressing precursor cells and express markers consistent with known enteroendocrine cell gene products. At the same time, serotonin cells in the corpus, also NeuroD-independent, express a number of mast cell markers and seemingly differentiate in an entirely separate manner from histamine cells.

The necessity of Ngn3 expression for endocrine differentiation in the stomach has been in question since the Ngn3-null mouse was produced. This mouse showed that hormones were still produced in the stomach without Ngn3, and three hormones were detectable: ghrelin, histamine, and serotonin (Jenny et al., 2002, Lee et al., 2002). Based

on the results of this study, we think it is likely that in Ngn3-deficient mice, the ghrelin and histamine cells are the Ngn3-independent cells observed by lineage tracing, while the serotonin cells are mast cells that have migrated to the corpus. Serotonin cells are seen in Ngn3-deficient stomach in reduced amounts, consisting of about one-sixth of the number of serotonin cells in wild-type neonatal stomach. This reduced number may reflect the early stage of development for the mouse, as the stomach does not fully develop until adulthood (past three weeks), and Ngn3 knockout neonates died 1-2 days postnatally. It is also likely demonstrative of the important role hormones play in the shaping of the gastric architecture, and the absence of enteroendocrine cells could alter the gastric epithelium such that mast cells have reduced capability to migrate to or reside in the epithelium (Fukushima et al., 2003).

The results of this study suggest that a higher percentage of antral serotonin cells derived from Ngn3-expressing precursors than previously described. Using the Ngn3-Cre mouse and a eYFP indicator mice, antral serotonin cells localize nearly 100% with eYFP, in contrast to the 30% shown previously using a LacZ indicator (Wang et al., 2007). While this is a significant departure from the previous finding, a population entirely arising from Ngn3-expressing precursors is logically consistent with the rest of this study's findings. Corpus serotonin cells are all but absent in mast cell-deficient mice, whereas antral serotonin cells are unaffected in number. This may be an indication that the eYFP indicator strain is more sensitive than β-galactosidase. Endogenous eYFP expression may be more sensitive than XGal staining, which is subject to pH conditions, or immunohistochemistry with β-galactosidase antibody. The presence of mast cells

producing serotonin in the corpus and gastric epithelial cells producing serotonin in the antrum appears to be a further distinguishing cellular difference between the corpus and antrum.

However, counts of various hormone populations with the eYFP indicator still do not stain all of the hormone cells. Even when using the seemingly more sensitive eYFP indicator strain, gastric corpus populations such as ghrelin and histamine are still only partially derived from Ngn3+ precursors. It is a possibility that Cre expression is mosaic in the stomach of these mice. Nonetheless, the existence of hormone expression in Ngn3-null mice is enough to demonstrate that some stomach endocrine cells do not require Ngn3 for differentiation, and lineage tracing results reaffirm this.

Lineage tracing studies showed that a large number of stomach endocrine cells do not express Ngn3 or NeuroD during their differentiation. While Cre recombination shows 30% of histamine-expressing cells derive from Ngn3-expressing precursors, it is possible that Cre recombination is incomplete in these cells. At the same time, the lineage tracing results showing absolute results - the absence of Ngn3 and NeuroD in serotonin cells and NeuroD alone in histamine cells - seem accurate when examined by RT-PCR.

The bone marrow transplant mouse model suggests that serotonin-expressing mast cells migrate from the bone marrow to the gastric corpus epithelium. Mice were only examined at three months post-engraftment due to the experimental conditions of the lab that generously provided the tissue, but further transplant experiments could elucidate the migration rate of these serotonin cells. Irradiated mice given GFP-labeled

donor bone marrow would be sacrificed at daily intervals starting immediately after transplantation, to measure the rate at which GFP-expressing serotonin cells appear in the gastric corpus epithelium. One drawback of the GFP bone marrow mouse is that irradiated mice given donor bone marrow do not appear to recapitulate wild-type conditions: the number of serotonin cells in the corpus of this mouse is five times greater than observed in wild type tissue. The irradiation itself may provoke a response that increases the migration or proliferation of mast cells. An inducible bone marrow labeling transgenic mouse model would show bone marrow migration without irradiation, and more closely recapitulate wild type migration and turnover.

The bone marrow model could be useful in extending this research into human tissue. While human and mouse stomachs are anatomically similar, there are notable differences, and there is currently no data to suggest human gastric serotonin cells are mast cells. Whether human gastric serotonin cells are bone marrow-derived mast cells could be tested in a method similar to the transgenic bone marrow mouse. Patients undergoing bone marrow transplants for blood cancers such as leukemia are often irradiated and given donor bone marrow from a healthy individual. In lieu of fluorescent labeling, donor bone marrow cells can be identified by in situ hybridization for the sex chromosomes, in cases of cross-gender bone marrow donations. Histological examination of the gastric epithelia of bone marrow transplant patients could demonstrate whether the same mast cell population resides in human stomach as in mouse stomach, and if it exhibits the same regional specificity.

The absence of NeuroD expression in HDC-CFP cells is puzzling when trying to understand how histamine cells differentiate. Ngn3 is present in at least a portion of these cells, but its downstream target NeuroD is absent. While ectopic expression Ngn3 is able to promote endocrine differentiation, it was thought to do this via NeuroD activation. One possible candidate for promoting NeuroD-independent endocrine differentiation is the zinc finger transcription factor Myt1b. Myt1b promotes endocrine differentiation in a Ngn3-dependent manner, and is expressed in HDC-CFP cells in preliminary data (Wang et al., 2008). The endocrine identity of HDC-CFP cells without NeuroD speaks to a nonessential or redundant role for NeuroD in these and possibly other endocrine cells.

While our RT-PCR results with histamine-expressing cells demonstrates that these cells do not express NeuroD, the question of how these cells differentiate in NeuroD's absence is still an open one. Histamine cells must still express some gene product, likely a transcription factor, which allows it to exit the cell cycle, and to activate expression of HDC. Rather than examining targeted individual transcripts for analysis, a high-throughput method such as deep sequencing would be able to identify numerous transcription factors. This method would also be more sensitive to relative expression levels, whereas our RT-PCR experiments focused primarily on candidate transcripts that could be classified as present or absent. Just as the serotonin cells which arose without Ngn3 expression were compared to a mixed population of endocrine cells using the Ngn3-Cre transgenic mouse, histamine cells could be compared to a population of NeuroD-derived endocrine cells isolated from the gastric corpus of a NeuroD-Cre;Rosa26-eYFP mouse.

The characterization of histamine-producing cells in this study has mainly demonstrated the absence of candidate markers. In light of the characterization of most histamine cells of the bone marrow as Gr1+/CD11b+ granulocytes rather than mast cells, it was important to exclude the possibility of these corpus histamine cells being granulocytic, rather than epithelial-derived endocrine cells. While the corpus histamine cells evidently lacked these cell surface markers by flow cytometry, it is still possible that these cells expressed these markers and lost their expression as a result of the epithelial microenvironment. But preliminary data using the GFP bone marrow donor mouse model also suggests that the histamine cells of the corpus do not derive from bone marrow, as anti-HDC antibody did not colocalize with GFP expression.

The existence of two very distinct serotonin populations in the stomach leads to the question of what roles do the two populations play. Serotonin has very wide-ranging effects on the immune system, nervous system, smooth muscle, digestive tract, and even bone formation (Bülbring and Crema, 1959, Cooke et al., 1997, Mossner and Lesch, 1998, Yadav et al., 2008). The function of serotonin is complex, given the identification of sixteen receptor subtypes (Hoyer et al., 2002). However, the presence of two serotonin populations in the stomach suggests that they may release serotonin in response to different stimuli. Typically, gastric endocrine cells secrete hormones in response to food intake stimuli, whereas mast cells secrete their products as part of immune response following IgE binding to FCεR1 (Lee et al., 2004). Expression in the gastric corpus of the mast cell receptor FCεR1, which signals for degranulation and release of serotonin in mast cells, would represent the strongest evidence for the two populations being distinct

in function. There are experimental methods to detect degranulation, such as annexin-V staining to detect exocytosing secretory granules, but they would be very technically challenging given the small number of available cells (Demo et al., 1999).

If the populations have different functions, then it is possible that mast cell-deficient mice, which lack the bone-marrow derived serotonin cells in the corpus, may have a phenotype specific to the stomach. Mast cell-deficient mice have been shown to be more susceptible to parasitic infection (Wada et al., 2010). A lack of corpus mast cells may cause an inability to mobilize immune cells in response to foreign contaminants in the stomach. The heterogeneity of mast cells found throughout the body means that there is a possibility that these gastric serotonin cells have gene products or functions unique among mast cells (Galli et al., 2005).

The other possibility is that bone marrow-derived serotonin cells and serotonin cells that arise from the gastric epithelium are identical or overlapping in function. In this case, the two derivations could either be a redundancy, or an evolutionary quirk. Complicated hormone signaling in the gut, much like the specialized portions of the gastrointestinal tract itself, are a trait of more advanced organisms. It may be that serotonin-producing cells developed in the gut as a more sensitive and specialized cell to take over functions previously provided by stomach-localized mast cells.

Serotonin released by the gastrointestinal tract is correlated to various diseases of the gut. Two notable diseases are irritable bowel syndrome (IBS) and carcinoid syndrome.

Irritable bowel syndrome (IBS) is a bowel disorder characterized by changed bowel habits accompanied by abdominal pain or discomfort. Serotonin is thought to play a role in the disease, and serotonin receptor antagonists are often used to treat it (Lomax et al., 2006). But studies on the level of serotonin in IBS patients show inconsistent findings (Bertrand and Bertrand, 2010, Lomax et al., 2006, Mawe et al., 2006). One possibility that comes with two distinct serotonin cell populations is that IBS could be caused by a change in one serotonin population in particular. This would not be obvious in counting overall serotonin cells or released serotonin. It would be a function of the size of one serotonin cell population, and of the cell's stimulus for degranulation.

Carcinoid tumors are a rare type of neuroendocrine tumor characterized by abundance of serotonin expression in the gut, which leads to flushing, diarrhea, and valvular heart disease. A cell of origin has not been identified, but the overexpression of serotonin in carcinoid patients has led researchers to believe that serotonin-producing cells are the cell of origin. The presence of a second, distinct serotonin cell population in the gastric epithelium could mean a secondary candidate for these tumors. These mast cells, while arising in bone marrow, reside in epithelial tissue, and literature suggests they express epithelial markers such as E-cadherin, which are important for their residing in host tissues (Nishida et al., 2003). Additionally, our preliminary experiments using epithelial markers did show expression of epithelial markers cytokeratin 18 and 19 by RT-PCR in corpus serotonin cells (unpublished observations). It is possible that a bone marrow cell that has a partial epithelial identity may give rise to an epithelial-like tumor. Another scenario could be that these mast cells are not yet terminally differentiated when

they arrive at the gastric epithelium and that they in fact retain a degree of plasticity. They may subsequently take on expression of epithelial markers due to signals from surrounding epithelial cells. Bone marrow cells have been shown to be recruited to differentiate into gastric epithelium in *Helicobacter*-infected stomach when the gastric stem cells are depleted (Houghton et al., 2004).

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