CHAPTER 3

REGULATION OF GASTRIC CORPUS EPITHELIAL CELL PROLIFERATION BY THE NOTCH SIGNALING PATHWAY

3.1: SUMMARY

The Notch signaling pathway is a key regulator of gastrointestinal epithelial cell homeostasis. Global inhibitor studies demonstrated a key role for Notch in regulating epithelial cell proliferation in the intestine and gastric antrum. In this study I investigated the mechanism by which Notch regulates epithelial cell dynamics in the gastric corpus by focusing on the function of the Notch1 and Notch2 receptors. Mice were treated with a pan-Notch inhibitor or inhibitory antibodies targeting Notch1 and/or Notch2. Epithelial proliferation was significantly decreased with all treatments, with Notch1+Notch2 treatment producing a similar decrease in proliferation to pan-Notch inhibition. Treatment of mouse and human corpus organoids resulted in a reduction in overall growth, demonstrating that Notch acts in an epithelial-specific manner. This study demonstrated that Notch signaling through the Notch1 and Notch2 receptors is required for regulating epithelial proliferation *in vivo* and *in vitro*.

3.2: INTRODUCTION

The epithelium of the gastric corpus is thought to be renewed by a small population of actively cycling stem cells. Stem cells generate progenitor cells that, upon exiting the stem cell niche, migrate bidirectionally to differentiate and generate the various differentiated epithelial cell lineages of the stomach. Few markers have been described to identify these stem and progenitor cells; they include *Lrig*[1] and *Sox2*[2] for actively-cycling corpus stem cells. *Troy*[3] has been described as a marker of quiescent stem cells in the gland base, and *Tff2*[4] as a committed progenitor giving rise to zymogenic and parietal cells.

Corpus glands can be divided into four regions: the pit, isthmus, neck, and base. Stem and progenitor cells are located in the isthmus[5]. The uppermost segment, the pit, houses surface mucous cells. Surface mucous cells contain mucous granules and express mucin 5AC (MUC5AC), gastrokine-1, and trefoil factor family 1 (TFF1)[6]. Parietal cells are the acid-secreting cells of the stomach, can be located in any region of the corpus gland, and express H/K ATPase (ATP4A)[6]. The pre-neck transition cells give rise to mucus-secreting mucous neck cells in the neck-region of the corpus gland, and eventually mature into digestive enzyme secreting chief cells base marked by gastric intrinsic factor (GIF)[7] in the mouse. Scattered throughout all regions are hormone-secreting enteroendocrine cells identified by chromogranin A (CHGA)[8]. The turnover rates of these cell types range from a few days for surface mucous cells, 2 months for parietal cells, to over 6 months for chief cells[9].

The Notch signaling pathway has been shown to be a key regulator of gastric stem cell homeostasis. In the antrum, genetic or pharmacological Notch inhibition led to

a decrease in proliferation of stem and progenitor cells and an increase in all differentiated cell types of the antrum[10,11]. Activation of Notch in Lgr5-positive stem cells resulted in an increase in proliferation, increased gland fission events, and gastric polyp formation[11]. Notch activation in parietal cells led to hyperproliferation and eventual adenoma formation[10]. Notch plays a key role in maintaining normal gastric homeostasis, but the mechanism through which Notch functions has not been identified.

Four Notch receptors (Notch1-4) exist in vertebrates that are single-pass transmembrane proteins[12]. Receptor signaling involves proteolytic cleavage of the receptor to release the Notch intracellular domain (NICD), which activates target gene transcription, such as those in the Hes and Hey gene families[13]. Notch1 and Notch2 are the primary receptors involved in intestinal stem cell homeostasis, with Notch1 having a predominant function[14–16]. In Chapter 2, I identified Notch1 and Notch2 as the primary receptors regulating gastric antral epithelial cell homeostasis.

Here I describe an analysis of the Notch receptors through which Notch regulates corpus epithelial cell homeostasis. Using inhibitory antibodies that detect mouse and human Notch1 and Notch2, I demonstrate that Notch1 and Notch2 are key regulators of epithelial cell proliferation both *in vivo* and *in vitro.* I also demonstrate that the Notch signaling pathway regulates human corpus stem cell function *in vitro*.

3.3: METHODS

Mice

Mice of both sexes, aged 2-3 months, were used. Notch1-CreERT2^{SAT} and Notch2-CreERT2^{SAT} [17], CBF:H2B-Venus[18](Jackson Labs #020942) and ROSA26-CAG-

LSL-tdTomato-WPRE (ROSA-Tomato)[19] (Jackson Labs #007909) mice have been previously described. All mice were on a C57BL/6 background except for CBF:H2B-Venus, which was on a mixed background (CD1 and FVB/NJ). Mice were housed under specific pathogen-free (SPF) conditions in automated watered and ventilated cage on a 12-hour light/dark cycle. All experimental protocols were approved by the University of Michigan Committee on the Use and Care of Animals.

Lineage-tracing

Notch1-CreERT2^{SAT}; ROSA-Tomato and Notch2-CreERT2^{SAT}; ROSA-Tomato mice were treated with 5 daily injections of Tamoxifen (TX; 1 mg/20g body weight), followed by a 2-week chase.

Notch Pathway Inhibition

For *in vivo* Notch inhibition, the gamma-secretase inhibitor dibenzazepine (DBZ, 30μmol/kg i.p., SYNCOM, Groningen, The Netherlands) or vehicle (0.1% Tween-80, 0.5% hydroxypropylmethylcellulose [E4M], 0.1% DMSO in water) was administered to mice once per day for 5 days, with stomach tissue collected the next day. Humanized IgG1 neutralizing monoclonal antibodies specific for the Notch1- or Notch2-negative regulatory region (referred to as N1 or N2), or an irrelevant control IgG1 antibody interacting with herpes simplex virus gD protein (referred to as Gd) were described previously[16]. Antibodies were injected i.p. at 5mg/kg on days 1 and 4, with collection of stomach tissue on day 6, 14, or 28.

For *in vitro* treatment of mouse and human organoids, gamma-secretase inhibitor DAPT (10μM; EMD4Biosciences, Gibbstown, NJ, USA) or Gd, N1, or N2 (10μg/mL) were added to culture media and renewed every other day for up to 5 days.

Tissue Collection and Histological Analysis

Mice were fasted overnight with free access to water before tissue collection. Stomachs were removed, opened along the greater curvature, fixed in 4% paraformaldehyde in PBS overnight at 4°C, and paraffin-embedded. Tissue sections were incubated with primary antibodies (Table 3-1) followed by appropriate secondary antibodies (1:400, Invitrogen, Grand Island, NY, USA) and sections were mounted with ProLong Gold containing 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen) as described previously[20]. Antigen retrieval was performed by Antigen Unmasking Solution (Vector), Trilogy (Cell Marque), or Tris-EDTA (10mM Tris Base, 1mM EDTA, 0.05% Tween-20, pH 9.0)(Table 3-1). Corpus glands were isolated and immunostained as previously described[11]. Imaging by digital microscopy was done using a Leica SP5X Inverted 2-Photon FLIM Confocal Microscope or a Nikon E-800 Microscope.

Table 3-1. Antibodies and lectins used for immunohistochemistry

Antibody	Species/Clonality	Source	Dilution	Antigen Retrieval*
GFP	Rabbit/Polyclonal	Invitrogen (A21311)	1:200	Antigen Unmasking Solution
Intrinsic Factor	Rabbit/Polyclonal	Dr. D Alpers, Washington University	1:1000	Antigen Unmasking Solution
RFP	Rabbit/Polyclonal	Rockland Immunochemicals (600-401-379)	1:200	Trilogy
E-cadherin	Rat/Monoclonal	Invitrogen (13-1900)	1:1000	Antigen Unmasking Solution
Mucin 5AC (Muc5AC)	Mouse/Monoclonal	Novocastra (NCL-HGM-45MI)	1:75	Antigen Unmasking Solution
Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1)	Rat IgG	BD Biosciences (557355)	1:1000	
α-Smooth muscle actin (αSMA)	Mouse/Monoclonal	Sigma-Aldrich (C6198)	1:500	
Chromogranin A (CHGA)	Rabbit/Polyclonal	Abcam (15160)	1:200	
Ki67	Rabbit/Monoclonal	Thermo Scientific (RM-9106)	1:200	Antigen Unmasking Solution
H/K-ATPase	Mouse/Monoclonal	MBL (D031-3)	1:1000	
Pepsinogen C (PepC)	Sheep/Polyclonal	Abcam (ab9013)	1:200	Tris-EDTA
Griffonia Simplicifolia II (GSII)	Lectin	Molecular Probes (L-21415)	1:1000	Antigen Unmasking Solution

*Antigen retrieval was performed on paraffin sections only with Antigen Unmasking Solution (Vector), Trilogy (Cell Marque), or Tris-EDTA (10mM Trizma Base, 1mM EDTA, 0.05% Tween-20, pH 9.0).

Gene Expression Analysis

RNA was isolated from gastric corpus tissue by homogenization in lysis buffer (RLT, Qiagen) with β-mercaptoethanol (10μL/mL) or from corpus organoids by passing organoids through a syringe with a 25G needle 20 times, followed by DNase I treatment and purification using the RNeasy Mini Kit (Qiagen). RNA was isolated from human corpus tissue using Trizol (Invitrogen), followed by DNase treatment and purification using the RNeasy Mini Kit (Qiagen). cDNA was prepared from 500ng total RNA and quantitative PCR was performed as described[21], using primer sets listed in Table 3-2.

Gastric Organoid Culture

Mouse corpus organoid culture was carried out as previously described[11]. In brief, culture conditions included resuspending glands in Matrigel and overlaying with culture media (50% L-WRN conditioned media, Advanced DMEM/F12, Pen-Strep, 20% FBS, L-Glutamine, 10nM Gastrin [Tocris]) with the addition of Y-27632 (Tocris,10μM) upon initial plating. Human gastric tissue was obtained under Institutional Review Board approved protocols and organoids were established as described[22] with modifications. Corpus tissue was incubated with 10mM dithiothreitol (DTT)(Invitrogen) in DPBS with Pen-Strep and Gentamycin for 15 min. at room temperature, followed by incubation in 12mM EDTA in DPBS with Pen-Strep and Gentamycin for 1 hour at 4°C on a rocking platform. Tissue was vigorously shaken for 1 minute to release glands, large tissue pieces were removed, and glands were pelleted at 100 x *g* for 5 minutes. Glands were plated in Matrigel as described for mouse gastric organoid culture. After 30 minutes incubation at 37^{o}C, culture media (50% L-WRN conditioned media, Advanced

Table 3-2. Oligonucleotide sequences used for qRT-PCR

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Product Size
Gapdh	TCAAGAAGGTGGTGAAGCAGG	TATTATGGGGGTCTGGGATGG	350
Notch1	AGCAAGAAGAAGCGGAGAGAGC	TGTCGTCCATCAGAGCACCATC	91
Notch2	TGTGGAAGGAATGGTGGCAGAG	CTCGGGCAGCAAGAAACAAAGG	194
Notch3	TGATCGGCTCTGTGGTGATGC	GGTGCTGTGTTCTCGCTTTCG	290
Notch4	ATTCCTCATGGGAAAGACA	ACTCCCATCACTACCACAAACC	91
H/K-ATPase	TGTACACATGAGAGTCCCCTTG	GAGTCTTCTCGTTTTCCACACC	157
Muc5AC	GCCGTGTCCAGGAGTCTAATACC	CAGCCTAGCCACCACCTTCAG	133
Gif	CTTGGCCCTGACCTGTATGT	TAGGTTGCTCAGGTGTCACG	191
Chga	AAGAAGAGGAGGAGGAAGAGG	TCCATCCACTGCCTGAGAG	149
*NOTCH1**	GACAGCCTCAACGGGTACAA	CACACGTAGCCACTGGTCAT	137
*NOTCH2**	CAACCGCCAGTGTGTTCAAG	GAGCCATGCTTACGCTTTCG	240
*NOTCH3**	TCTTGCTGCTGGTCATTCTC	TGCCTCATCCTCTTCAGTTG	485
*NOTCH4**	TGAGGTGAATCCAGACAAC	ATACAGTCATCCAGGTTCTC	261
*ACTB**	CATCGAGCACGGCATCGTCA	TAGCACAGCCTGGATAGCAAC	211

*Sequences designed to amplify the human gene; all other primers amplify the mouse gene

DMEM/F12, Pen-Strep, 20% FBS, L-Glutamine, Gastrin [10nM, Tocris], Y-27632 [10μM], and SB431542 [10 μM, Tocris Bioscience]) was added to each well. Media was renewed every other day.

Morphometrics

Morphometric analysis was performed with ImageJ software (1.46r, Wayne Rasband, NIH, USA). For analysis of proliferating Ki67 cells, the entire length of the corpus for each animal was imaged (n=4-6 animals per group) and cell counts were normalized to epithelial length (μm).

Statistics

GraphPad Prism software was used for statistical analysis. Quantitative data are presented as mean ± SEM and analyzed using Student's t-test or 1-way ANOVA with Dunnett's *post-hoc* test, as indicated. qRT-PCR data experiments are expressed as mRNA fold change vs. control (vehicle or Gd, as stated) with $P<0.05$ considered significant.

3.4: RESULTS

Notch regulates epithelial proliferation in the corpus

Pan-Notch inhibition has previously been shown to reduce corpus epithelial proliferation[10]. In order to better characterize how Notch is regulating proliferation, I focused on the function of individual Notch receptors. Gene expression analysis of the four Notch receptors (*Notch1-4*) indicated *Notch1* and *Notch2* are the most highly

expressed receptors in both full-thickness and epithelial corpus tissue, with lower expression of *Notch3* (Fig 3-1).

To test the function of the NOTCH1 and NOTCH2 receptors in regulating this effect, I examined proliferation after treatment with inhibitory antibodies that selectively target NOTCH1 or NOTCH2 and compared the cellular effects to pan-Notch inhibition with the gamma-secretase inhibitor DBZ. Analysis of Ki67-positive cells showed a marked reduction in cellular proliferation with combined receptor inhibition to an extent similar to global Notch inhibition by DBZ (Fig 3-2 A-F). Treatment with anti-NOTCH1 (N1) or anti-NOTCH2 (N2) alone led to a significantly reduced number of proliferating cells, but to a lesser extent than N1+N2 (Fig 3-2D-H). Morbidity of the N1+N2 and DBZ group prevented analysis of tissues past 6 days [16,23]. This indicates that Notch1 and Notch2 are key regulators of corpus epithelial proliferation.

Notch regulates corpus organoid growth

I established *in vitro* mouse corpus organoids to test if the proliferation effect of Notch inhibition *in vivo* is due to Notch signaling intrinsic to corpus epithelial cells. To test if there was Notch signaling in corpus organoids, I established organoids from the *CBF:H2B-Venus* mouse. The *CBF:H2B-Venus* mouse provides a fluorescent cell readout of Notch signaling through the nuclear binding of NICD with CBF/RBPJκ[18]. Organoids established from the *CBF:H2B-Venus* Notch reporter mouse showed Venus expression, demonstrating active Notch signaling in corpus organoid culture (Fig 3-3A). Gene expression profiling showed that, similar to what was observed in corpus glands, Notch1 and Notch2 were the primary Notch receptors expressed in corpus organoids

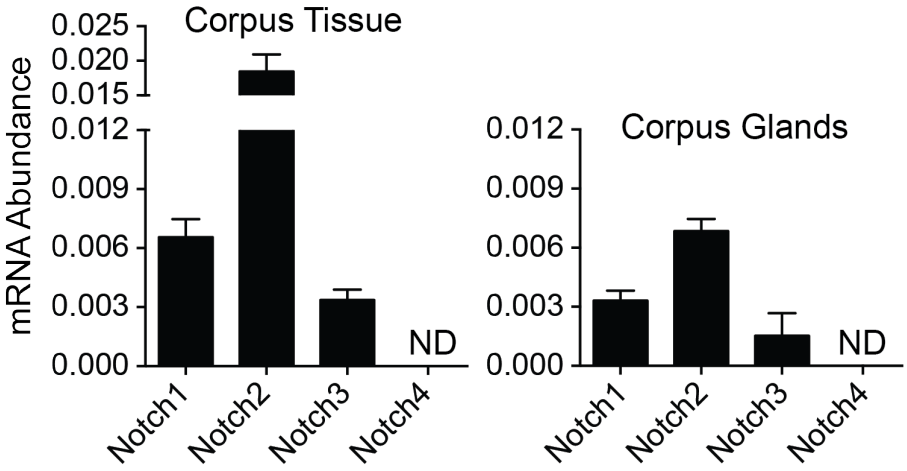

Figure 3-1. Gene expression of Notch receptors in the mouse corpus. Notch receptor expression was determined by qRT-PCR analysis of total RNA isolated from full-thickness corpus tissue or corpus glands (mean ± SEM n=3-5 mice).

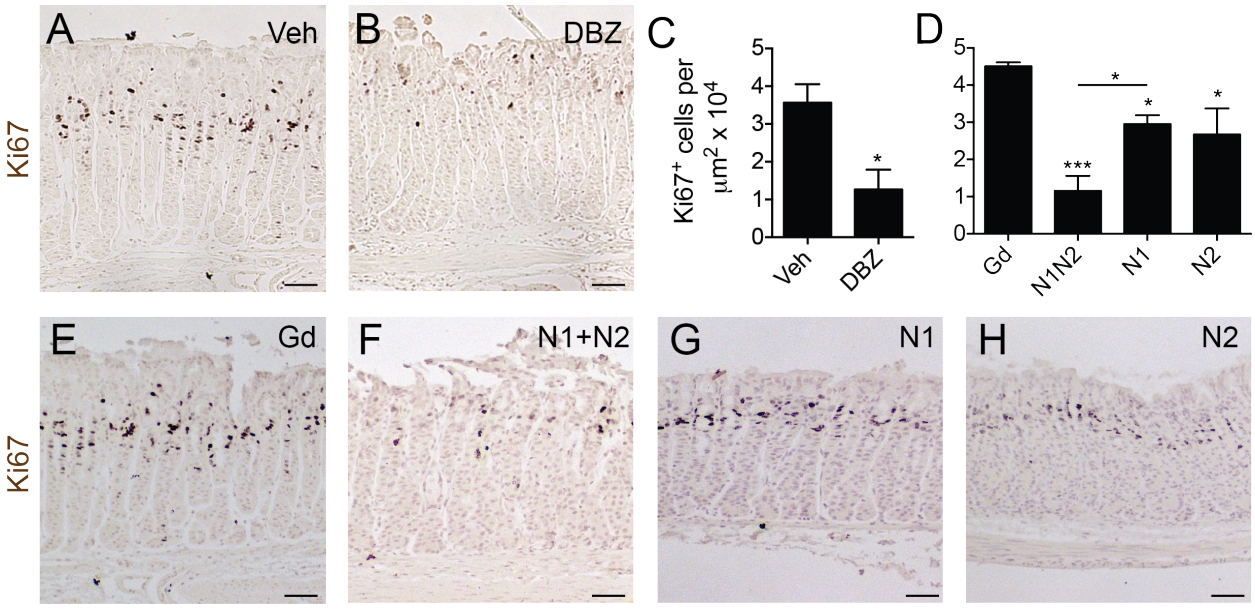

Figure 3-2. Inhibiting Notch signaling components reduces epithelial proliferation. Paraffin sections from (A) Vehicle, (B) DBZ, (E) Gd antibody control, (F) anti-NOTCH1+anti-NOTCH-2, (G) anti-NOTCH1, and (H) anti-NOTCH2 were stained for proliferating cells by Ki67 incorporation. Morphometric analysis of Ki67(C,D) stained cells (mean ± SEM; n=3-5 mice). *P<0.05, ***P<0.001 vs. Vehicle using Student's t-test or vs. Gd using 1-way ANOVA. Scale bars: 50µm.

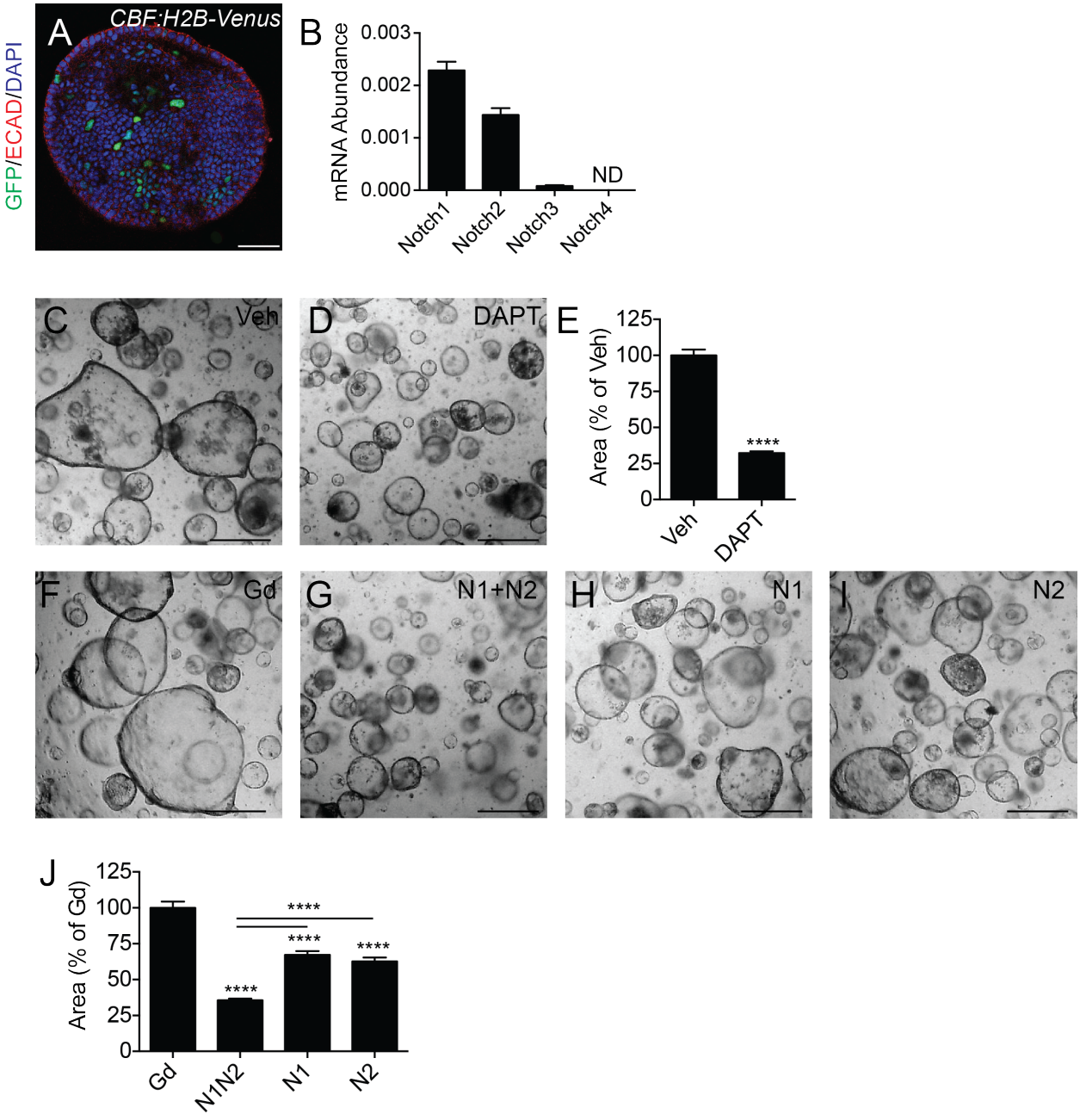

Figure 3-3. Notch regulates mouse corpus organoid growth. (A) Corpus organoids established from the CBF:H2B-Venus Notch reporter mouse strain. (B) Notch receptor expression was determined by qRT-PCR analysis of total RNA isolated from corpus organoids (mean ± SEM n=3 independent lines), Morphology (C,D,F-I) and quantitative measure of size (E,J) of corpus organoids following 5 days of treatment with (C) vehicle (DMSO), (D) DAPT, (F) Gd, (G) anti-NOTCH1+anti-NOTCH2, (H) anti-NOTCH1, or (I) anti-NOTCH2 (mean ± SEM; n=3 wells). *P<0.05, **P<0.01, ****P<0.0001 vs. Vehicle using Student's t-test or vs. Gd using 1-way ANOVA. Scale bar = (A) 50μm or (B,C,E-H) 250μm. ND = Not detected.

(Fig 3-3B). Notable in contrast to isolated glands, expression of Notch3 was negligible in corpus organoids.

To investigate Notch function in regulating corpus organoid growth, organoids were treated with the pan-Notch inhibitor DAPT, and reduced overall growth was observed (Fig 3-3C-E). Reduced growth was also observed after receptor targeting, with combined N1+N2 inhibition reduced to a similar extent as pan-Notch inhibition. Individual receptor targeting mimicked what is seen *in vivo,* with significant growth reduction, but to a lesser extent than N1+N2 (Fig 3-3F-J). These findings demonstrate that Notch signaling is present in organoid culture and that intrinsic Notch signaling through NOTCH1 and NOTCH2 is required for mouse corpus organoid growth.

Notch is necessary for growth of human corpus organoids

I took advantage of the design of the Notch receptor inhibitory antibodies to detect both human and mouse receptors to test the regulation of human corpus stem cells *in vitro* by NOTCH1 and NOTCH2. I established organoids from human corpus tissue (Fig 3-4, Table 3-3) and identified the expression of the Notch receptors in both human corpus tissue (3 patients) and established human corpus organoids (2 patient lines). Similar to the mouse, *Notch1* and *Notch2* were the primary receptors expressed in both human corpus tissue and organoids (Fig 3-5A). There was significant variation in Notch receptor expression levels in patient tissues and organoids, described in Fig 3-6.

I found that intrinsic Notch signaling was required for growth of human corpus organoids, with a reduction in overall organoid growth observed after treatment with DAPT (Fig 3-5B-D). Reduced growth was also observed after receptor targeting, with

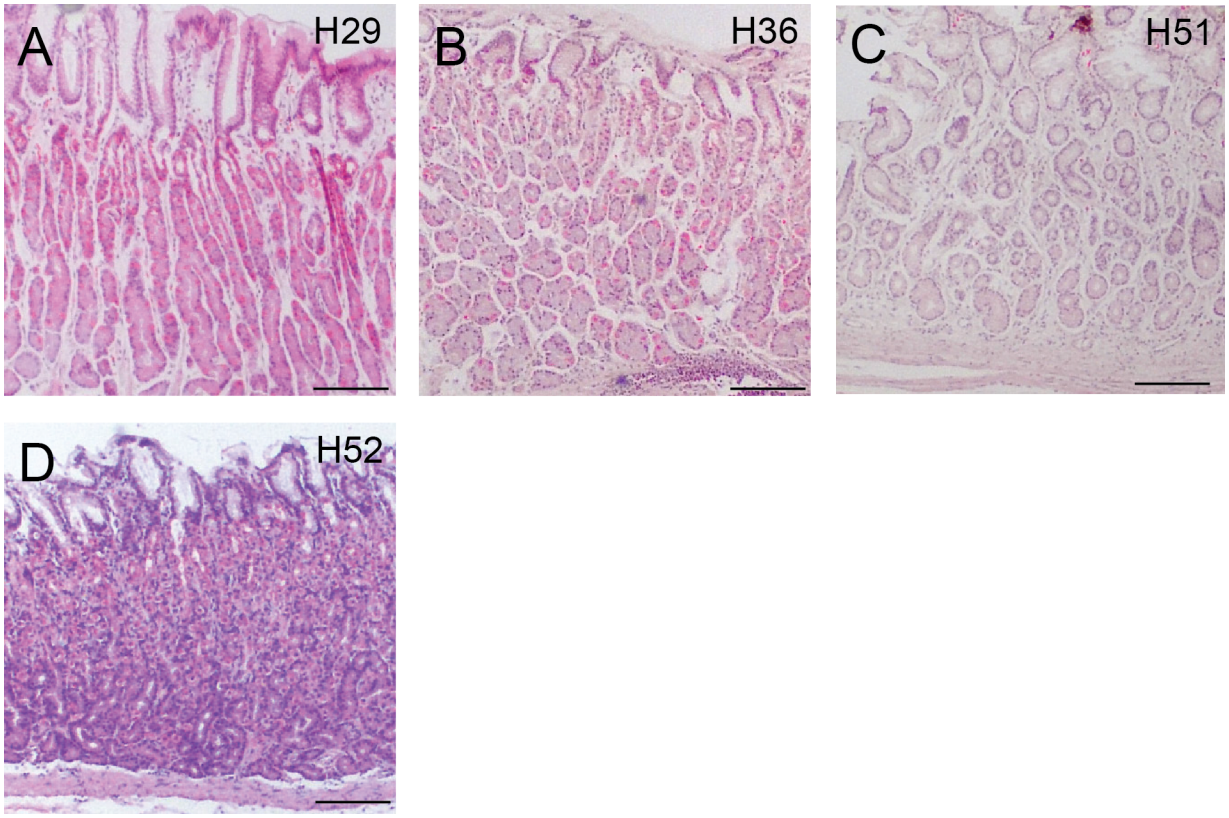

Figure 3-4. Histology of human gastric tissue used in study. (A-D) Haematoxylin and eosin stained sections of human tissues used for organoids or RNA. Note: Patient H45 was a biopsy, and no histology is available. Scale = 200μm.

Table 3-3. List of patient information for human tissues

Patient	Age	Gender	Tissue Type
H29	59	M	Surgical
H36	56	M	Surgical
H45	48	F	Biopsy
H51	56	M	Surgical
H52	48	M	Surgical

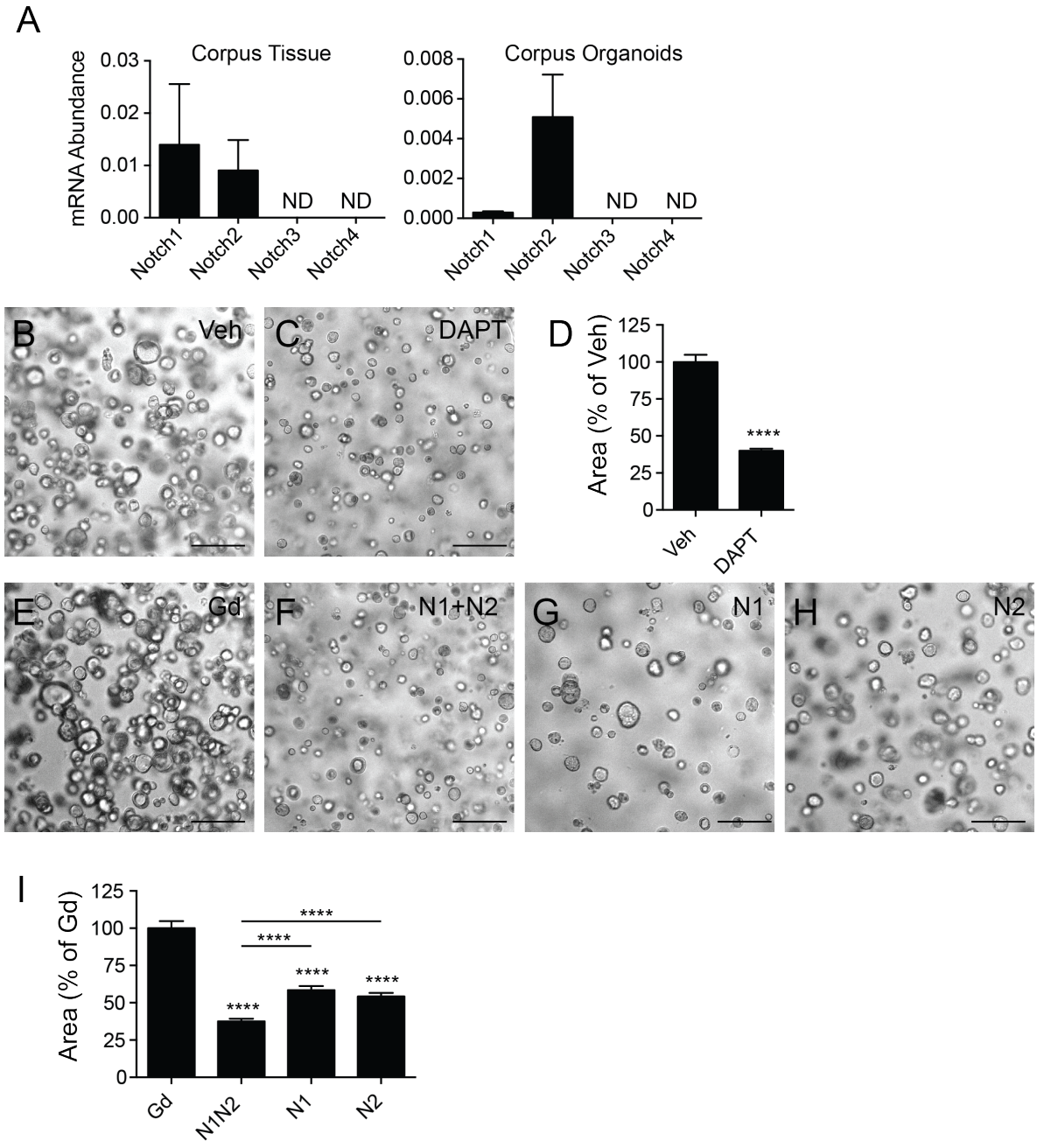

Figure 3-5. Human corpus organoid growth is regulated by Notch signaling. (A) Gene expression of Notch receptors in full thickness human corpus tissue or human corpus organoids was determined by qRT-PCR (mean ± SEM, n=3 patients, n=2 organoid line). Morphology (B,C,E-H) and quantitative measure (D,I) of size of organoids following treatment with (B) vehicle, (C) DAPT, (E) Gd, (F) anti-NOTCH1+anti-NOTCH2, (G) anti-NOTCH1, or (H) anti-NOTCH2 (mean ± SEM, n=4 wells, 100 organoids per well). **P<0.01 vs Vehicle using Student's t-test, ***P<0.001, ****P<0.0001 vs Gd using 1-way ANOVA. Scale = 100μm. ND = Not detected.

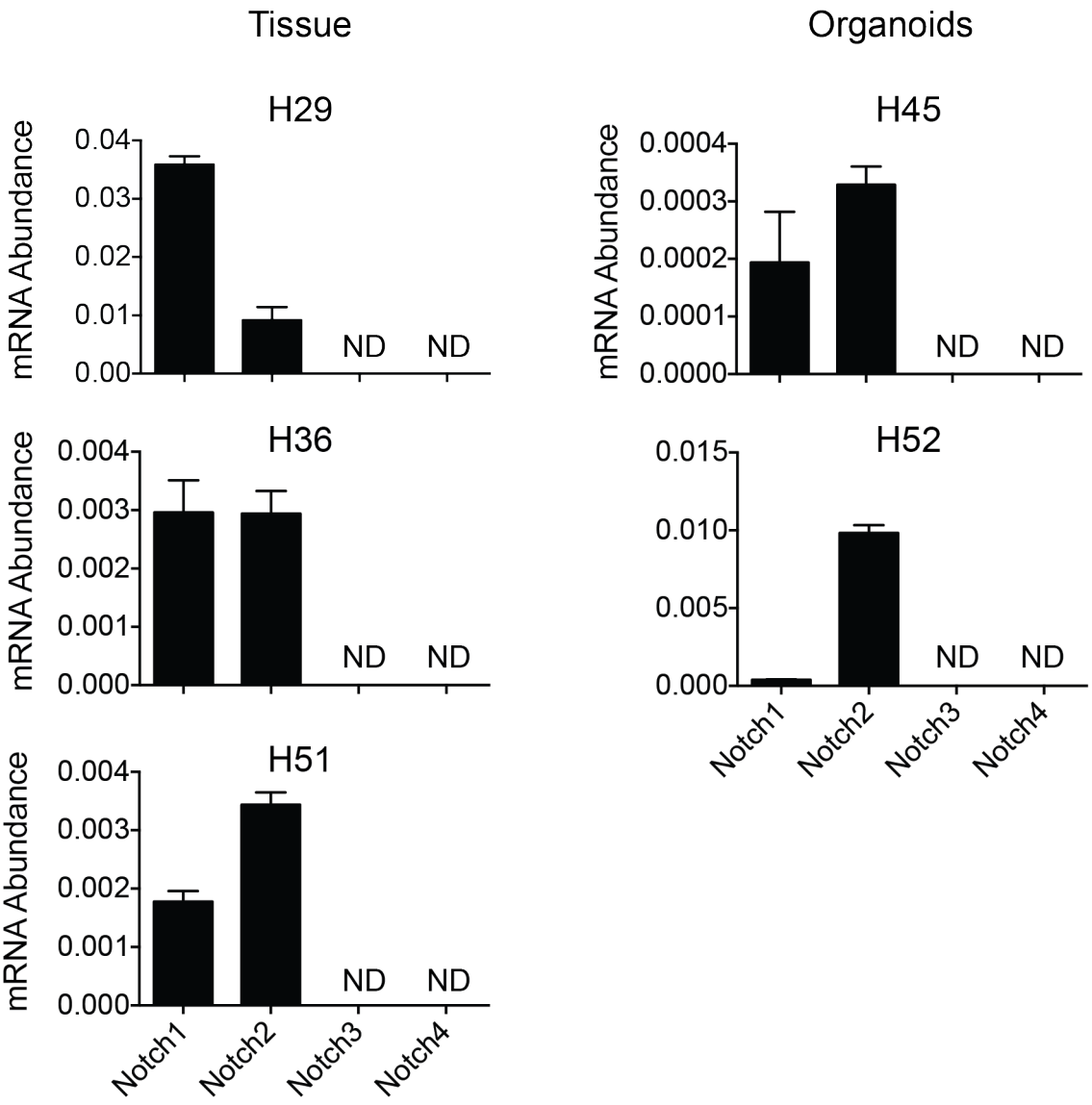

Figure 3-6. Variable level of Notch receptor expression from human corpus tissue. Expression of Notch receptors from individual patient tissues or organoid lines (mean ± SEM; n=3 technical replicates). ND = Not detected.

combined N1+N2 inhibition similar to pan-Notch inhibition. Individual receptor targeting resulted in an intermediate growth reduction, similar to what was observed with mouse corpus organoids (Fig 3-5E-I). This finding suggests that NOTCH1 and NOTCH2 play key roles in regulating human corpus organoid growth.

Notch signaling in the corpus epithelium

Due to the significant role Notch signaling plays on proliferation of epithelial cells, I wanted to analyze if differentiated cells were also altered with inhibition of Notch signaling. I first identified which cell types were expressing active Notch signaling as well as the NOTCH1 or NOTCH2 receptors. To identify cells undergoing Notch signaling *in vivo* in the gastric corpus I utilized the *CBF:H2B-Venus* Notch reporter mouse. Isolated corpus glands showed scattered Venus-positive cells throughout the length of the gland (Fig 3-7A). These Venus-positive cells co-stained with an antibody directed to the pan-endocrine marker CHGA (Fig 3-7B), and H/K ATPase, a marker of parietal cells (Fig 3-7C). In addition to epithelial cell labeling, there was extensive non-epithelial cell Notch signaling observed in paraffin tissue sections similar to what I observed in the antrum (Chapter 2). Co-staining demonstrated that the majority of the Venus-positive stromal cells undergoing active Notch signaling are PECAM-1-expressing endothelial cells (Fig 3-8A) and α-Smooth muscle actin smooth muscle cells (Fig 3-8B).

To test if NOTCH1 and NOTCH2 are expressed in stem or proliferative cells in the corpus, I performed a lineage tracing experiment using mice expressing Notch receptor-CreERT2 fusion genes crossed to ROSA-Tomato mice. We examined N1-

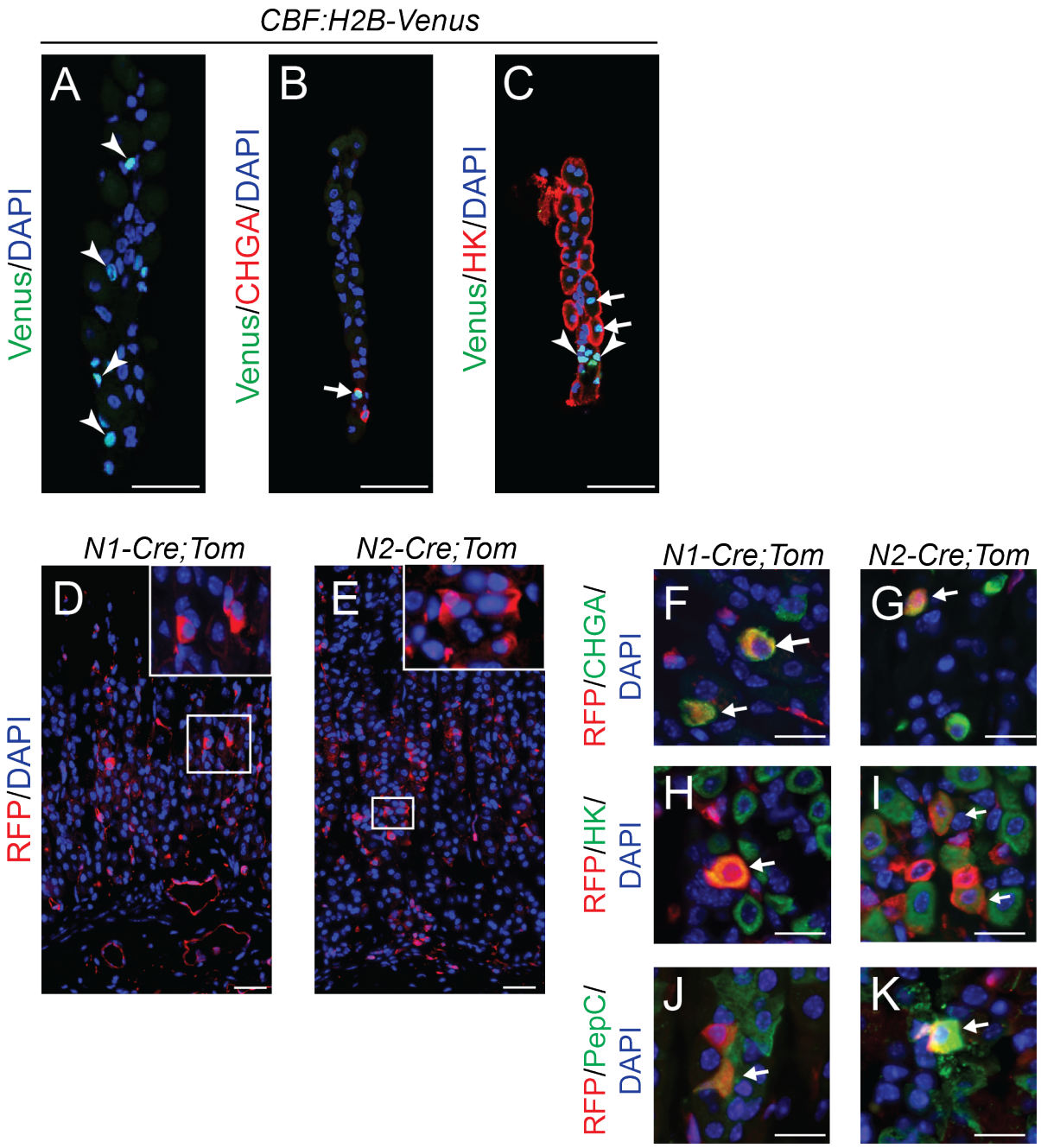

Figure 3-7. Notch signaling is present in the mouse gastric corpus. (A) Corpus gland isolated from CBF:H2B-Venus mice (green, nuclear) co-stained with (B) the pan-endothelial cell marker CHGA or (C) parietal cell marker H/K ATPase. Paraffin sections from (D) N1-CreERT2;Tomato and (E) N2-CreERT2;Tomato mice were stained for RFP (red) with DAPI (blue) nuclear counterstain 2 weeks after Cre activation. N1-Cre;Tom and N2-Cre;Tom sections were co-stained with (F,G) endocrine cell marker CHGA, (H,I) parietal cell marker H/K-ATPase (HK), or (J,K) zymogenic cell marker Pepsinogen C (PepC). Scale bar: 50μm (A-E) or 25μm (F-K).

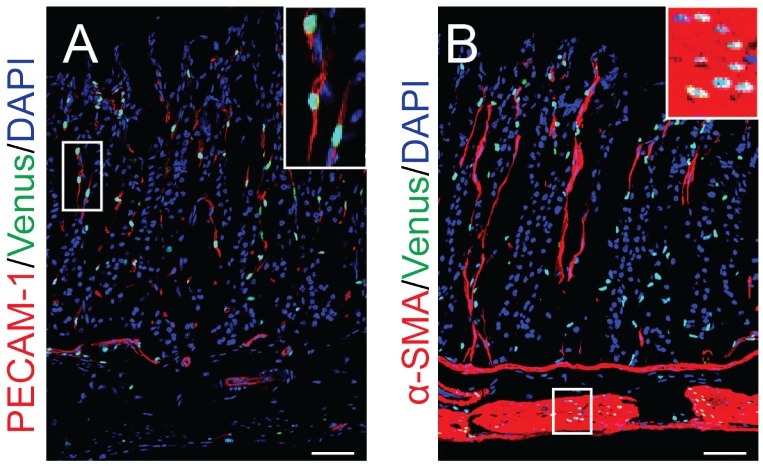

Figure 3-8. Notch signaling is present in non-epithelial cells in the gastric corpus. Frozen tissue sections from CBF:H2B-Venus mice were co-stained with (A) the endothelial marker PECAM-1 (red) or (B) the smooth muscle cell marker α smooth muscle actin (α-SMA) (red). Scale bar: 50μm.

CreERT2^{SAT};ROSA26-Tomato (N1Cre;Tom) and N2-CreERT2^{SAT};ROSA26-Tomato (N2Cre;Tom) mice 2 weeks post-tamoxifen. Tomato expression from Notch1 and Notch2 was prevalent in single cells in the corpus (Fig 3-7D,E). Unlike the antrum (Chapter 2, Fig 2-1) and intestine[17], no lineage stripes were observed from either Notch1 or Notch2. Instead, single epithelial and non-epithelial cells had Tomato expression. To identify which cells express Notch1 or Notch2, co-staining of the Tomato-positive cells indicated that Notch1 and Notch2 are present in enteroendocrine (Fig 3-7F,G), parietal (Fig 3-7H,I), and chief (Fig 3-7J,K) cells.

Notch regulation of corpus epithelial differentiation

Notch signaling is a key regulator of cell fate decisions in the intestine and gastric antrum[11,24,25]. Thus, I analyzed the role of Notch signaling for the homeostasis of differentiated cell types in the corpus epithelium following pan-Notch inhibition or specific Notch receptor inhibition. Analysis of parietal (Fig 3-9A-D), enteroendocrine (Fig 3-9E-H), chief and deep mucous (Fig 3-9I-L), and surface mucous (Fig 3-9M-P) cells by gene expression and immunohistochemistry did not show any significant alteration of differentiated cell types following inhibition of the Notch pathway. One hypothesis as to why no changes to differentiated cell types were observed *in vivo* despite NOTCH1 and NOTCH2 expression in differentiated cell types is the short time-point available for analysis due to morbidity of animals. Turnover time of cell types in the corpus is significantly longer than the intestine or antrum, with parietal cells requiring 2 months for turnover and chief cells turning over in 6 months.

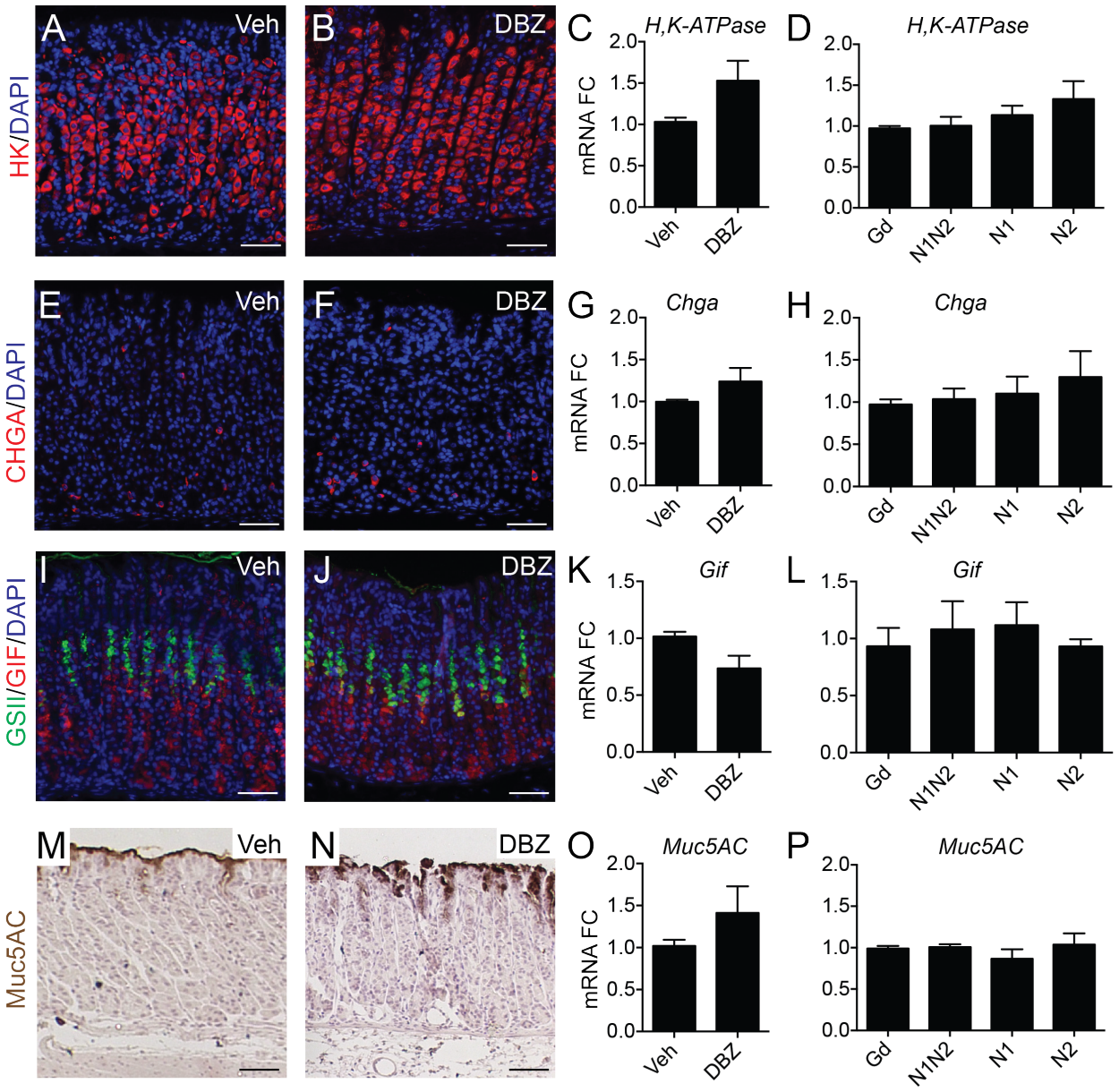

Figure 3-9. Notch inhibition does not alter differentiated cell types. Immunohistochemistry and qRT-PCR analysis of (A-D) Parietal, (E-H) enteroendocrine, (I-L) deep mucous (GSII) and chief (GIF), and (M-P) surface mucous cells. qRT-PCR analysis of markers of differentiated cells with global Notch inhibition (C,G,K,O) or Notch receptor inhibition (D,H,L,P). Scale = 50 μm.

The short time-point available for Notch inhibition does not encompass the required turnover time for these cell types in the corpus. However, surface mucous cells turnover in 3-5 days, and so further investigation is needed as to why I do not observe changes to surface mucous cells with Notch inhibition in the corpus.

3.5: DISCUSSION

Here I report that Notch signaling regulates gastric corpus epithelial cell proliferation through the NOTCH1 and NOTCH2 receptors. The inhibition of both NOTCH1 and NOTCH2 mimics global Notch inhibition through DBZ in respect to epithelial proliferation *in vivo*. This effect is also seen with Notch inhibition of mouse corpus organoids, with DAPT or NOTCH1 and NOTCH2 inhibition showing a similar reduction in organoid growth. Furthermore, I have shown that human corpus stem cells are regulated by Notch signaling, with Notch inhibition by DAPT or NOTCH1 and NOTCH2 of human corpus organoids leading to a reduction of organoid growth. This identifies the Notch signaling pathway, specifically through NOTCH1 and NOTCH2, as a key regulator of mouse and human corpus epithelial cell proliferation.

The development of methods to grow epithelial organoids from primary mouse and human tissue is a powerful approach to investigate gastric stem cell regulation[22,26,27]. The role of NOTCH1 and NOTCH2 in regulating corpus epithelial proliferation was similar *in vitro* and *in vivo*, with inhibition of NOTCH1 or NOTCH2 alone causing a significant reduction in growth/proliferation but not to the extent seen with pan-Notch inhibition or N1+N2 inhibition. This validates the use of *in vitro* organoids for studying corpus epithelial dynamics. Furthermore these studies showed that Notch

signaling is intrinsic to the gastric epithelium, with Notch signaling required for organoid growth.

Understanding the role of Notch receptors in the regulation of gastrointestinal epithelial homeostasis allows for a better understanding of the mechanism of Notch signaling and how it may be disrupted in pathologies such as gastric cancer. *NOTCH2*, *NOTCH2*, and *NOTCH3* are all up-regulated in gastric cancer cell lines and gastric carcinomas[28,29](Table 1-3). Activation of Notch signaling in parietal cells eventually leads to the formation of adenomas in the corpus[10]. Because this and other studies have shown that Notch has a profound effect on regulation of proliferation in the corpus, further studies are needed to understand the contribution of Notch over-activation in the development of hyperproliferative pathologies. By identifying the functional role of NOTCH1 and NOTCH2 we can better understand how the misregulation of these Notch components can contribute to disease formation.

Though NOTCH1 and NOTCH2 are expressed in enteroendocrine, parietal, and chief cells, short-term Notch inhibition did not lead to any changes in differentiated cell markers. The lack of effect on differentiated cells is not surprising given the lengthy turnover rate for corpus differentiated cells. Parietal cells turnover every 54 days and chief cells turnover every 194 days[9]. However surface mucous cells turnover in 3-5 days, and so the lack of alteration to markers of surface mucous cells with Notch inhibition may suggest that Notch does not influence the differentiation of this cell type. Thus the 6-day timepoint that I was limited by for Notch inhibition did not encompass the extended turnover times required for corpus epithelial cells. The 6-days Notch inhibition timepoint is due to animals becoming moribund with global Notch inhibition after 7

days[16,24], likely due to intestinal toxicity. In comparison, antral epithelial cells undergo turnover in 7-10 days, and all differentiated cell types exhibited a significant up-regulation with Notch inhibition[11,26]. This highlights the need to identify a gastric-specific Cre-driver to be able to genetically manipulate the Notch signaling pathway in the stomach without affecting the intestine. This would allow extended timepoints to further study Notch function in the gastric corpus. One promising mouse model is the *Sox2-Cre* mouse which is expressed in the stomach, but not intestine[2]. This mouse could be crossed to genetic mouse models of Notch inhibition, such as *Rbpj*$^{floxed/floxed}$ [30] or *Notch1*$^{floxed/floxed}$ and *Notch2*$^{floxed/floxed}$ mice (Jackson Lab no. 007181 and 010525) to manipulate Notch pathway in the stomach. Importantly this approach will allow investigation of longer timepoints of Notch inactivation by avoiding intestinal-associated morbidity.

In conclusion the Notch signaling pathway is key to regulating epithelial proliferation in the stomach through the NOTCH1 and NOTCH2 receptors. *In vitro* experiments identify Notch signaling as intrinsic to the gastric epithelium, and inhibition of Notch reduces growth of mouse and human corpus organoids. Further investigation is needed to understand the role of Notch signaling in cell fate choice in the corpus epithelium.

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CHAPTER 4

MOUSE AND HUMAN GASTRIC ORGANOID CULTURE

4.1: SUMMARY

Recent studies have highlighted the role of stem cells for epithelial turnover along the gastrointestinal tract. An *in vitro* culture system has been developed to culture stem cells in order to study mechanisms regulating stem cell homeostasis, proliferate and generate cell types of the epithelium. Stem cells in culture form *in vitro* epithelial spheroids, or organoids, that mimic features and cell types of the *in vivo* epithelium. These organoids are generated in the absence of underlying mesenchymal influence and thus are a powerful tool to study epithelial-specific cellular processes. This chapter details the protocols that I established to generate gastric organoids from mouse and human tissue. The organoids exhibited properties similar to the *in vivo* epithelium, such as tight junctions, cellular polarity, and proliferating and differentiated cells. Notch pathway inhibition showed a key role for this pathway to regulate organoid growth and stem cell function. Organoids were established from human gastric normal and tumor tissue, showing more rapid establishment and growth rates for tumor organoids.

4.2: INTRODUCTION

A recent development of culture methods allows for the study of epithelial cell regulation. Key advances in defining specific growth factors necessary for maintenance of tissue stem cells as well as identifying an adequate extracellular matrix has led to the establishment of organoids from numerous tissues. Mouse epithelial-specific organoids have been cultured from the liver[1], pancreas[2], Barrett's esophagus[3], stomach[4], small intestine[5], and colon[3,6].

A physiologically relevant long-term intestinal culture was from the intestine of neonatal mice grown in a collagen gel using an air-liquid interface[7]. These cultures formed cystic spheres containing both epithelial and mesenchymal cells, composed of proliferative and differentiated cell types. However, the culture system failed to recapitulate the crypt-villus architecture of the adult intestine and could not be robustly generated from adult tissue[7]

Sato and colleagues introduced an alternative culture system where they established intestinal organoids from isolated epithelial stem cells[5]. This mesenchyme-independent system allowed for long-term growth and expansion of epithelial organoids. Organoids had morphological features similar to the adult small intestine, such as specific crypt and villus domains, and exhibit cellular proliferation, differentiation, and apoptosis that mimics *in vivo* cellular homeostasis[5].

The method established by Sato allowed for the long-lived culture of organoids by defining the minimal requirements for sustaining growth of intestinal stem cells. Intestinal crypts or sorted Lgr5+ stem cells were resuspended in Matrigel, a laminin and collagen-rich matrix secreted by the cells that acts as a basement membrane

substitute[5]. The minimal required growth factors for intestinal organoid establishment and maintenance includes epidermal growth factor (EGF), R-spondin, and Noggin. R-spondin is a Wnt signaling enhancer and physiologic ligand of LGR5. Wnt is a key pathway to maintain stem cell homeostasis and drive cellular proliferation[8]. EGF exhibits a strong mitogenic effect on stem cells and the Ras/Raf/Erk/Mek signaling axis is required for *in vivo* stem cell homeostasis[9]. BMP signaling is active in the intestinal crypts and acts to inhibit intestinal stem cell renewal through suppression of the Wnt signaling pathway[10]. The BMP inhibitor Noggin allows for formation of crypt-like structures that bred outward from the spheres. Previous studies indicated that Noggin creates a crypt-permissive environment[11]. This is the basic makeup of media requirements for intestinal organoid formation from intestinal crypts. Organoids derived from isolated Lgr5+ intestinal stem cells were shown to have a higher rate of establishment when an inhibitor of Rho-associated protein kinase (ROCK) is added to prevent anoikis[5]. This methodology of intestinal organoid development has served as a base for development of organoids in other tissue types, including the stomach[4].

The establishment of organoids from the mouse stomach is very similar to the protocol used for the mouse intestine. Gastric glands are dissociated by incubating gastric tissue fragments in EDTA followed by mechanical disruption and resuspension of glands in Matrigel. In the original report, the distinguishing factor between mouse gastric and intestinal organoids is the strict dependence of gastric organoids on Wnt3a[4]. Moreover, FGF10 was required to drive budding events around the central lumen. Single antral Lgr5+ cells were able to establish organoids at an average efficiency of 9%[4].

An alternative mouse corpus organoid culture condition was introduced by the Zavros lab that more effectively generates mature gastric cell lineages[12]. Gastric glands were isolated and resuspended in Matrigel, but they were grown in co-culture with immortalized stomach mesenchymal cells (ISMCs). These corpus organoids maintained robust numbers of surface and deep mucous, chief, enteroendocrine, and parietal cells, with decreased proliferating cells compared to organoids grown without ISMC co-culture. This study showed the first example of a gastric organoid culture system that reflected the cell types and function of native tissue[12] .

Gastrointestinal organoids are established as cystic structures with a single cell layer surrounding a central lumen. Cells retain normal physiological turnover and are sloughed into the lumen every 2-3 days[5]. The organoids require passaging every week by mechanical disruption to relieve build-up of apoptotic cells in the lumen and are able to rapidly reform[5,13]. Organoids derived from the three regions of the small intestine and two regions of the stomach maintain their regional identity *in vitro*[4,12,14].

The establishment of long-lived epithelial organoids from gastrointestinal stem cells has had a significant impact on the study of gastrointestinal stem cell dynamics. Recently the impact of the organoid system has been applied to investigation of human gastrointestinal biology. Gastrointestinal organoid culture of human tissue is based on the mouse culture conditions with the requirement of R-spondin, EGF, and Noggin, with key modifications. Currently there have been two studies showing successful establishment of organoids from human gastric tissue, and they differ significantly in the required media compositions[15,16].

The Clevers' lab has successfully published studies establishing gastric organoids from human tissue[15]. In addition to Wnt, R-spondin, and Noggin, human gastric organoids require the addition of FGF10, a TGFβ inhibitor (A83-01), nicotinamide, a p38 inhibitor (SB202190), Wnt inducer glycogensynthase-kinase 3β inhibitor (CHIR99021) and a ROCK inhibitor (Y-27632)[15]. These conditions produce long-lived gastrointestinal spheroid cultures that exhibit features of the mouse stomach.

An alternative human gastric culture condition was published by the Stappenbeck lab that utilized Wnt3a, R-spondin-3, and Noggin conditioned media with addition of a ROCK inhibitor (Y-27632) and TGFβR1 inhibitor (SB431542)[16]. Reduction of Wnt3a, R-spondin-3, and Noggin together with the addition of the γ-secretase inhibitor DAPT was used to induce differentiation of the cultures. This culture technique was widely applied to biopsies of healthy patients and patients with inflammatory diseases from Barret's esophagus, stomach, small intestine, and colon to produce long-lived organoids[16].

This chapter outlines the development of the organoid technique for mouse and human gastric tissues for use in the Samuelson lab. This includes overall growth and maintenance of organoids, organoid characteristics, and the role that Notch signaling plays in gastric organoid homeostasis. We also compare and contrast human gastric organoid growth utilizing the two different media conditions, described above[15,16].

4.3: METHODS

Animals

Wild-type mice of both sexes aged 2-3 months were used. All mice were on a C57BL/6 background. Mice were housed under specific pathogen-free conditions in automated watered and ventilated cages on a 12-hour light/dark cycle. All experimental protocols were approved by the University of Michigan Committee on the Use and Care of Animals.

Mouse Organoid Establishment Protocol

Mouse gastric organoid culture was carried out as previously described with modifications[4,17]. Gastric tissue was incubated in 15 mM EDTA/Dulbecco's Phosphate-Buffered Saline (DPBS) with Penicillin-Streptomycin (pen-strep) for 1 h at 4°C on a rocking platform. Tissue was vortexed for 2.5 min to release glands, which were gravity-settled 2 × 5 min to remove large tissue chunks, and glands were collected by centrifugation at 150× *g* for 10 min. Glands were resuspended in Matrigel and plated in the center of wells in a 24-well plate in 40µl aliquots with approximately 200 glands per aliquot. After 30 min at 37^{o}C, culture media was added to each well: advanced DMEM/F12 with HEPES, 10% Wnt3A-conditioned media[4] and 5% R-spondin-2-conditioned media[18], B27, L-Glutamine (2mM), N2, Pen-Strep, Noggin (100 ng/ml; R&D Systems), EGF (50 ng/ml; Invitrogen) and Y-27632 (10 µM; Tocris). In some experiments, 50% L-WRN-conditioned media[17] replaced Wnt3A- and, R-spondin2-conditioned media, Noggin and EGF. Conditioned media was harvested from Wnt3a secreting L cells, Rspondin-2 secreting HEK293 cells, or WRN-secreting L cells, as previously described[4,17,18]. Media compositions are described in Table 4-1. Media was renewed every 2-3 days.

Human Organoid Establishment Protocol

Full thickness human gastric tissue and gastric biopsies were obtained under Institutional Review Board approved protocols. Human organoid establishment was carried out as previously described with modifications[16]. Gastric tissue (surgical resection, Gift of Life, or biopsy tissues) was incubated with 10mM dithiothreitol (DTT; Invitrogen) in DPBS with Pen-Strep and Gentamycin for 15 min at room temperature, followed by incubation in 12mM EDTA in DPBS with Pen-Strep and Gentamycin for 1 hr at 4°C on a rocking platform. Tissue was vigorously shaken for 1 min to release glands, large tissue chunks were removed, and gland were pelleted at 100 x *g* for 5 min. Tumor tissue was minced finely and pelleted. Glands were densely resuspended in an appropriate volume of Matrigel and plated in a 24-well plate well. After 30 min at 37^{o}C, culture media was added to each well. Two different media compositions were tested for organoid formation. Media A includes 25% Wnt3a conditioned media[4], 25% R-spondin2 conditioned media[18], Advanced DMEM/F12, 2mM L-Glutamine, B27, N2, HEPES, Pen-Strep, 1mM N-acetyl-cysteine, 10mM Nicotinamide, Noggin (100ng/mL; R&D Systems), EGF (50ng/mL; Invitrogen), A-83-01 (0.5 μM; Tocris), SB202190 (10 μM; Sigma-Aldrich), Chiron 99021 (2.5 μM; Tocris), and Thiazovivin (2.5 μM; Selleck Chemicals). Media B includes 50% L-WRN conditioned media[17], Advanced DMEM/F12, 1X Pen-Strep, 10% FBS, 2 mM L-Glutamine, 10μM Y-27632 and SB431542 [10 μM, Tocris Bioscience]). Media compositions are described in Table 4-1. Media was renewed every other day.

Table 4-1. Media compositions for mouse and human organoids.

Media	Composition
Mouse Media A	Advanced DMEM/F12 10% Wnt3A Conditioned Media[4] 5% R-spondin2 Conditioned Media[18] B27 N2 Pen-Strep L-Glutamine (2mM) Noggin (100ng/mL) EGF (50ng/mL) Y-27632 (10μM)
Mouse Media B	Advanced DMEM/F12 50% L-WRN Conditioned Media[17] FBS (10% for antrum, 20% for corpus) Pen-Strep L-Glutamine (2mM) Y-27632 (10μM)
Human Media A	Advanced DMEM/F12 25% Wnt3A Conditioned Media[4] 25% R-spondin2 Conditioned Media[18] L-Glutamine (2mM) B27 N2 HEPES Pen-Strep N-acetyl-cysteine (1mM) Nicotinamide (10mM) Noggin (100ng/mL) EGF (50ng/mL) A-83-01 (0.5μM) SB202190 (10μM) Chiron 99028 (2.5μM) Thiazovivin (2.5 μM)
Human Media B	Advanced DMEM/F12 50% L-WRN Conditioned Media[17] 10% FBS Pen-Strep L-Glutamine (2mM) Y-27632 (10μM) SB431542 (10μM)

Organoid Maintenance

Mouse and human organoids were split every 4-6 days. For splitting, Matrigel was removed from organoids by resuspending patty in PBS, organoids were incubated in Trypsin-EDTA (0.25%) at 37°C for 2 minutes, then vigorously pipetted 40 times. Organoids were re-plated at a density of 1:2 (human) or 1:3 (mouse).

Organoid Fixation and Embedding

For sectioning and immunostaining, Matrigel was removed from organoids by resuspending patty in PBS and organoids were fixed in 4% paraformaldehyde for 20 minutes. Organoids were stained with 0.2% methylene blue to help visualize organoids in the embedding process, embedded in OCT and frozen. Sections were cut at 4µm.

Organoid Immunostaining

For frozen sections, organoids were incubated with permeabilization/blocking solution (0.5% Triton X-100, 5% donkey or goat serum) for 2 hours at 37°C. Primary and secondary antibodies were diluted in staining solution (0.05% Tween 20, 5% serum) overnight at 4°C or for 30 minutes at room temperature, respectively (Table 4-2). ProLong Gold + DAPI was added to stained organoid sections.

For whole-mount staining, matrigel was removed and organoids were fixed in 2% PFA for 30 minutes, pelleted at 300 x *g* for 5 minutes, and resuspended in blocking solution (10% donkey or goat serum, 0.2% Triton X-100, 0.1% BSA, 0.05% Tween-20) for 30 minutes. Primary and secondary antibodies were applied in suspension (Table 4-2). Stained organoids were re-suspended in DPBS and mounted on slides with ProLong

Table 4-2. Antibodies used for immunohistochemistry.

Antibody	Species/Clonality	Source	Dilution
E-cadherin	Rat/Monoclonal	Invitrogen (13-1900)	1:1000
ZO1	Rabbit/Polyclonal	Zymed (40-2200)	1:500
GFP	Chicken/Polyclonal	Abcam (ab13970)	1:200
Gastrin	Rabbit/Polyclonal	DAKO (A0568)	1:1000
H/K-ATPase	Mouse/Monoclonal	MBL (D031-3)	1:1000
Ki67	Rabbit/Monoclonal	Thermo Scientific (RM-9106)	1:200
Griffonia Simplicifolia II (GSII)	Lectin	Molecular Probes (L-21415)	1:1000

Gold + DAPI. To measure proliferation, EdU (10μM) was added to the media for 1.5 hours before collection.

Notch Pathway Inhibition

For *in vitro* treatment of mouse and human antral organoids, the γ-secretase inhibitor DAPT (1μM antrum, 10 μM corpus; EMD4Biosciences, Gibbstown, NJ, USA) was added to media every other day for up to 5 days. For a re-establishment to assess stem cell function, organoids were treated with 500nM of DAPT for 2-5 days and the ability of organoids to re-form after passaging assessed.

Statistics

GraphPad Prism software was used for statistical analysis. Quantitative data are presented as mean ± SEM and analyzed using Student's t-test or 1-way ANOVA with Dunnett's *post-hoc* test, as indicated. qRT-PCR data are expressed as mRNA fold change vs. control with $P<0.05$ considered significant.

4.4: RESULTS

Mouse Gastric Organoid Establishment and Characterization

The technique used to establish organoids from the mouse stomach was adapted from previously published protocols[4,17]. A summary of the mouse gastric organoid protocol is depicted in Fig 4-1. After isolated glands were plated in Matrigel, spheres formed from glands within 6-hours. The efficiency of sphere formation was >70% from antral glands and >40% from corpus glands. Mouse organoids were characterized by a sphere that expanded in size over time in culture with smaller budding structures occurring after 4 days in culture following establishment or passaging (Fig 4-2A). After 6 days in culture, organoids exhibited a darkened lumen due to the accumulation of dead cells, which can be seen in a section stained by H&E (Fig 4-2B).

Mouse gastric organoids grow as a single layer of polarized epithelial cells, shown by E-cadherin (Fig 4-3A,B) and ZO1 (Fig 4-3C) staining. Mouse organoids are primarily comprised of proliferative cells, shown by Ki67 or EdU staining in antral (Fig 4-3D,E,G) and corpus (Fig 4-3H) organoids. Expression of differentiated cell markers, such as gastrin-positive enteroendocrine cells in antral organoids (Fig 4-3F) and H/K-ATPase-positive parietal cells in corpus organoids (Fig 4-3I) was demonstrated by immunostaining. Gene expression analysis of gastric organoids indicates the presence of enteroendocrine, zymogenic, and mucous cells in antral organoids (Chapter 2; Fig 2-5,7).

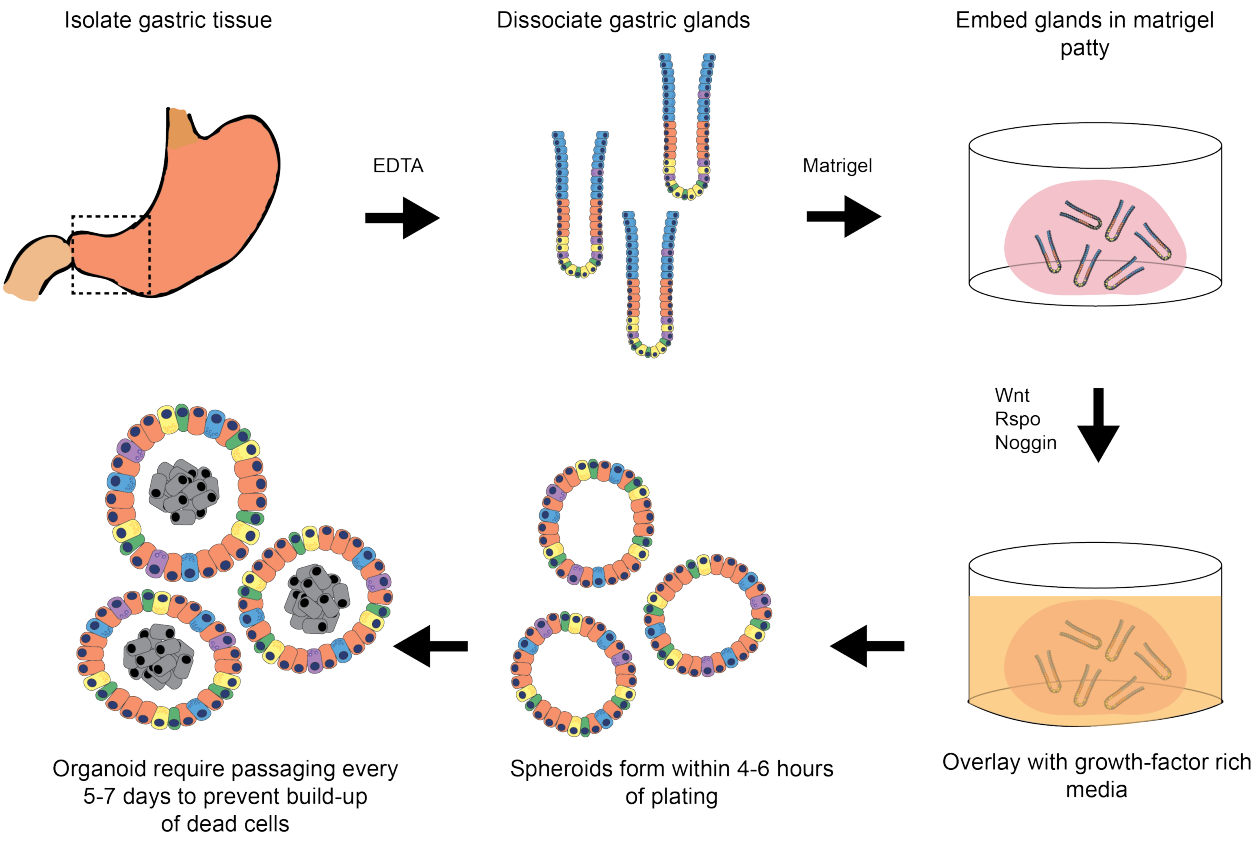

Figure 4-1. Summary of gastric organoid establishment. Glands are isolated from gastric tissue and resuspended in matrigel. After matrigel has polymerized into a half-sphere, media is added that contains Wnt, R-spondin (Rspo), and Noggin, as well as other growth factors and small molecules. Spheroids form from isolated glands within 4-6 hours and require passaging every 5-7 days.

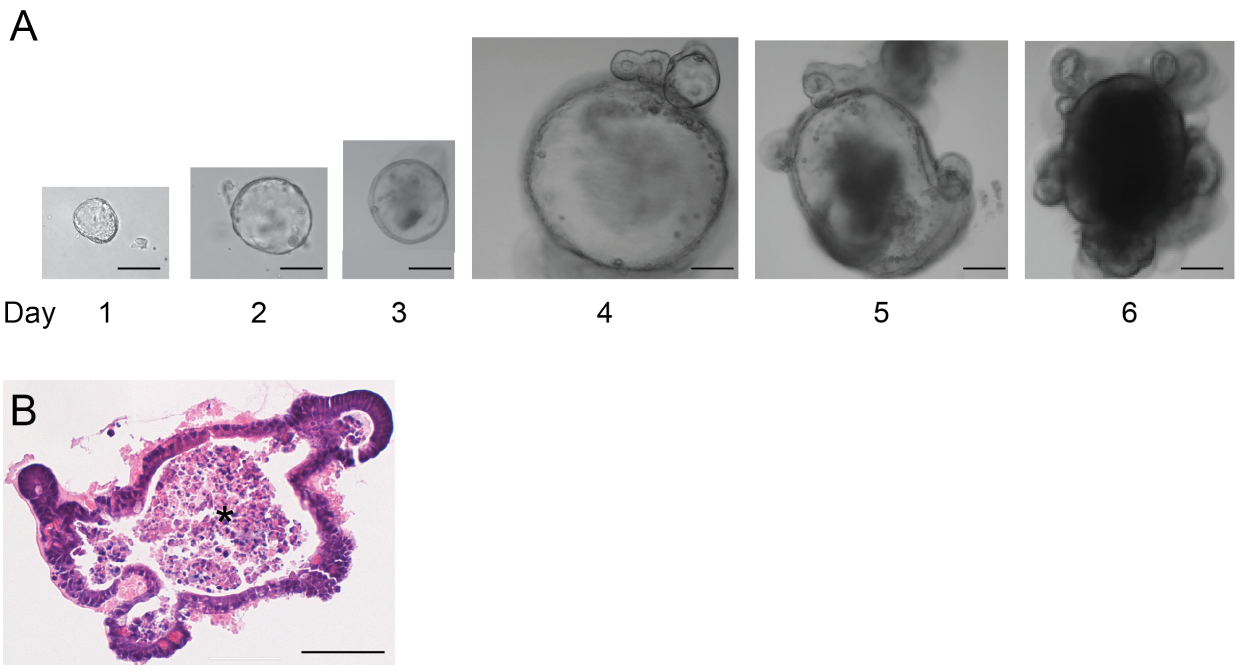

Figure 4-2. Mouse gastric organoid growth. (A) Mouse antral organoid growth over 6 days following passage. (B) H&E stained section of 5-day old mouse antral organoid. Scale = 100μm. *Dead cells.

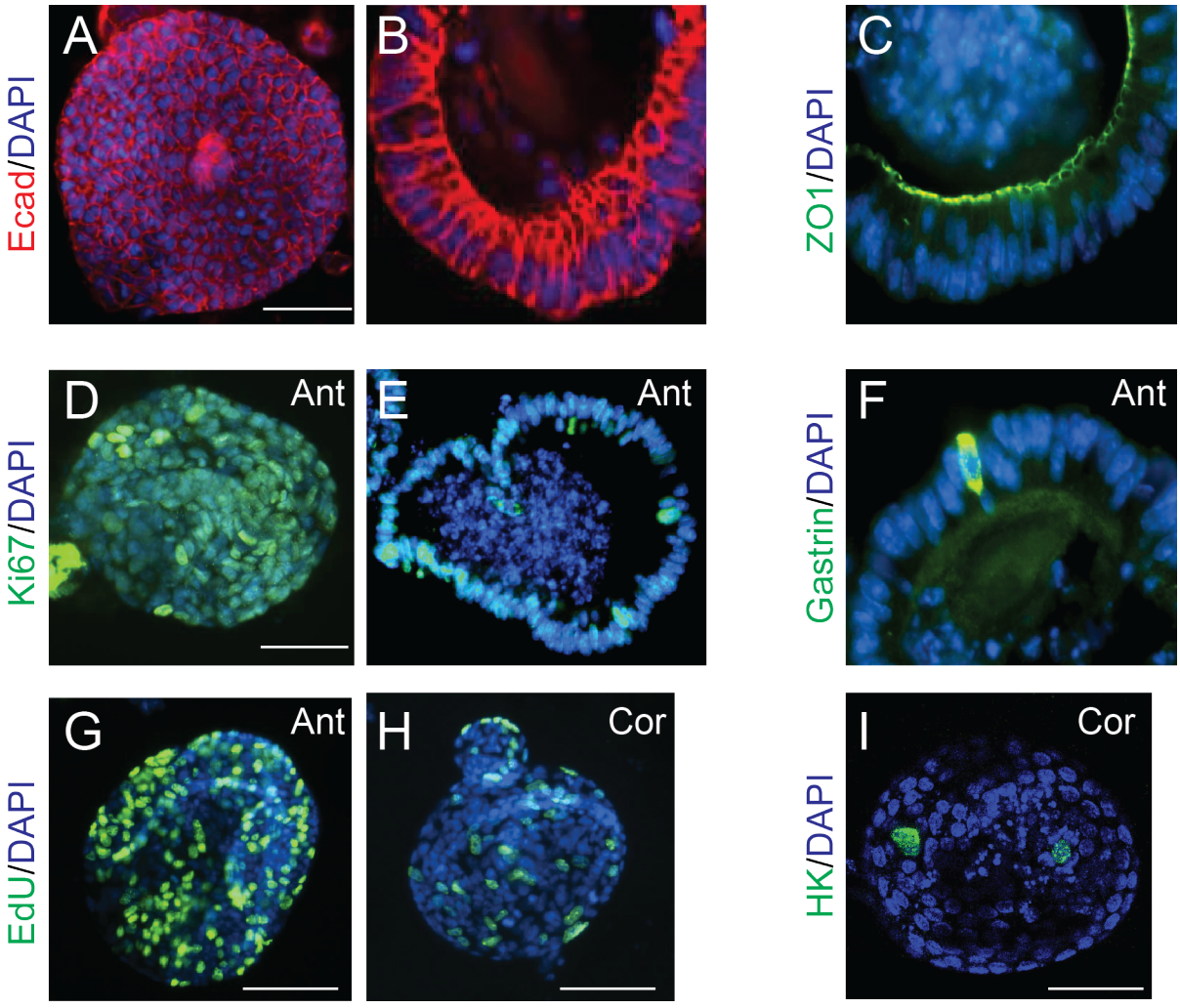

Figure 4-3: Characterization of mouse gastric organoid histology. (A,B) Mouse organoids are composed of polarized epithelial cells shown by E-cadherin (Ecad) (red) and (C) ZO-1 (green) immunohistochemical staining with DAPI nuclear counterstain (blue). (D,E) Organoids exhibit numerous proliferating cells marked by Ki67 (green). (F) Rare gastrin expressing G-cells (green) were observed. (G,H) EdU incorporation shows high levels of proliferation in (G) antrum (Ant) and (H) corpus (Cor) organoids. (I) Corpus organoids express rare H/K-ATPase-positive Parietal cells. Scale = 100 μM.

Regulation of Mouse Gastric Organoids by Notch Signaling

To test the role of Notch signaling on maintenance of gastric organoids, a dose-response analysis was performed by adding increasing concentrations of the γ-secretase inhibitor DAPT to organoid culture media for 5 days and assessing overall organoid growth on day 6 by measuring organoid size. For antral organoids, significant inhibition of growth was observed with 100nM of DAPT and organoid death was observed starting at 5μM of DAPT (Fig 4-4A, data not shown). Images of organoid treated with vehicle (Fig 4-4B-D) or 500nM DAPT (Fig 4-4E-G) showed a smaller overall size with DAPT treatment. Although Notch inhibition reduced growth, the cells appeared to be intact, as shown by E-cadherin immunostaining (Fig 4-4H,J). Proliferation was examined by examining by EdU incorporation, demonstrating a reduction in labeling in DAPT-treated organoids (Fig 4-4K) compared to vehicle-treated (Fig 4-4I).

To assess the affect of Notch inhibition on antral organoid stem cell function, a re-establishment assay was performed. Organoids were pre-treated with 500nM DAPT for 1-5 days followed by passaging and assessing the number of organoids that were able to re-form compared to vehicle-treated. Decrease organoid re-forming capability was observed with 1 day of DAPT treatment, with a significant inhibition seen with 3 or more days of DAPT treatment (Fig 4-5). This finding indicates that Notch inhibition affected stem cell function.

Corpus organoids are significantly less sensitive to Notch-inhibition, with a modest growth reduction seen with 500nM DAPT, but robust growth inhibition seen with 10μM DAPT, 10-fold higher concentration than antral organoids (Fig 4-6A). A decrease in overall size

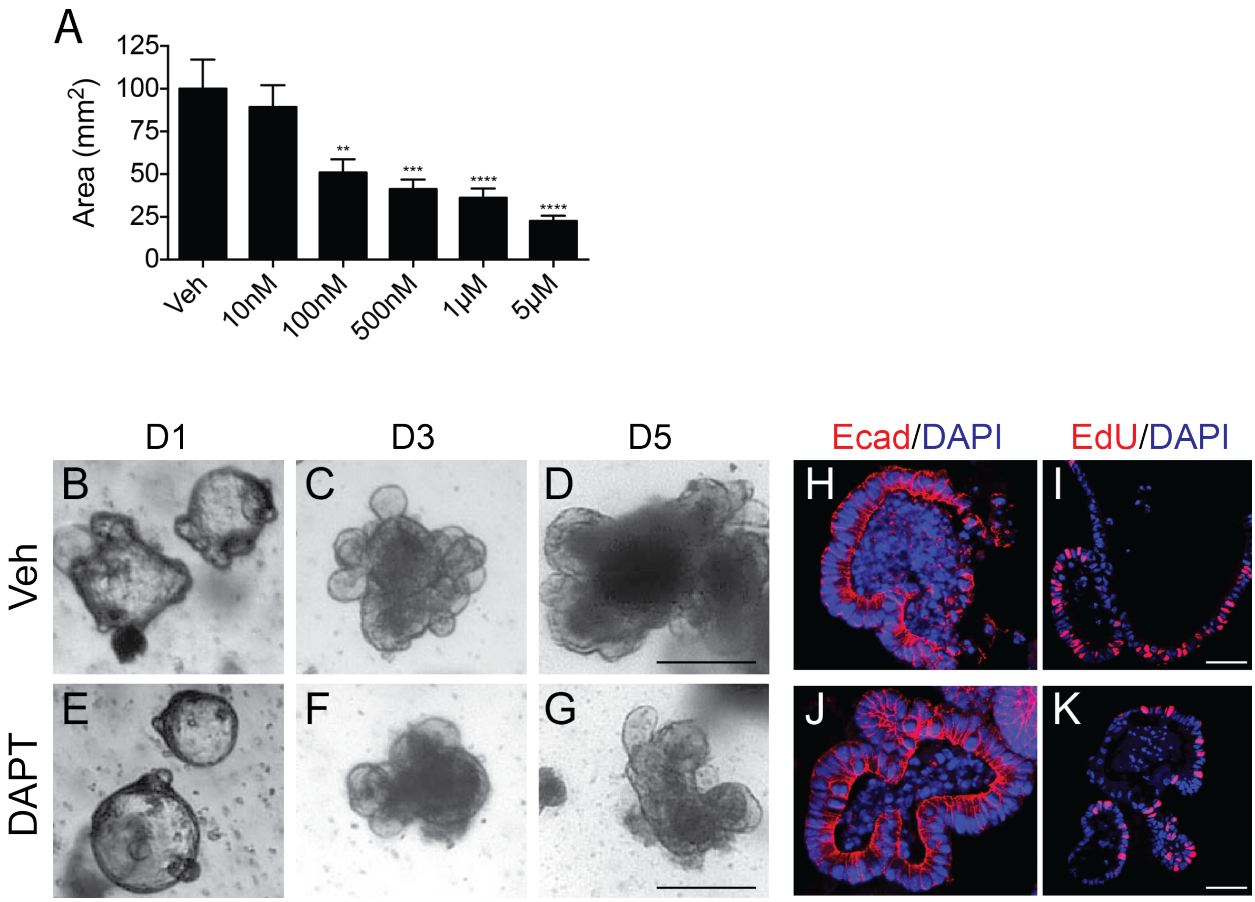

Figure 4-4. Notch Regulation of mouse gastric antral organoid growth. (A) Organoid growth was measured on day 6 after 5 days of treatment with the γ-secretase inhibitor DAPT (mean ± SEM, n=50 organoids per treatment). Representative images of (B-D) vehicle or (E-G) DAPT (500nM) treated organoids. (H,J) E-cadherin staining showed intact epithelium in (H) vehicle and (J) DAPT-treated organoids. Proliferating cells were marked by EdU incorporation in (I) vehicle and (K) DAPT-treated organoids. Scale = 50 μm.**P<0.01, ***P0.001, ****P<0.0001 vs. Veh using 1-way ANOVA. Adapted from Demitrack et al.[19].

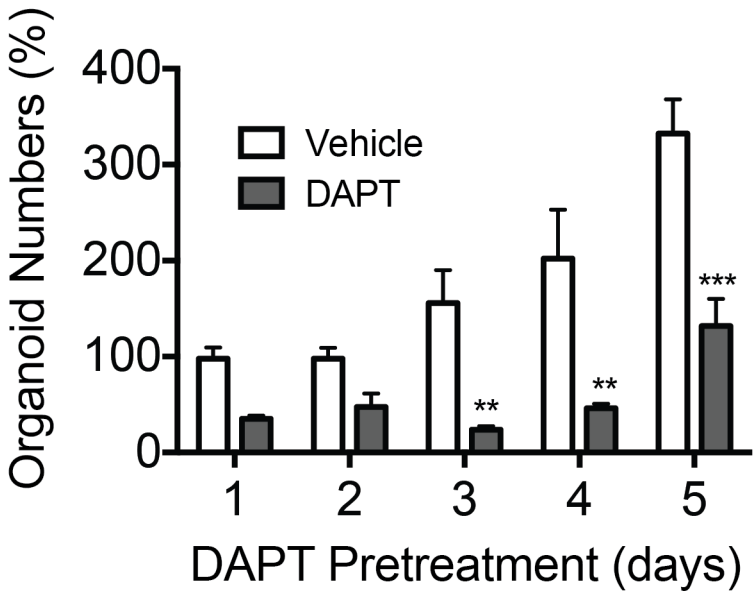

Figure 4-5: Notch inhibition reduces antral stem cell function in organoids. Antral organoid stem cell function was analyzed by assessing the re-plating efficiency of organoids 48 hours after passaging following 1-5 days of DAPT treatment (500nM) compared to pre-passage number of organoids. **P<0.01, ***P<0.001 vs. Vehicle using 1-way ANOVA.

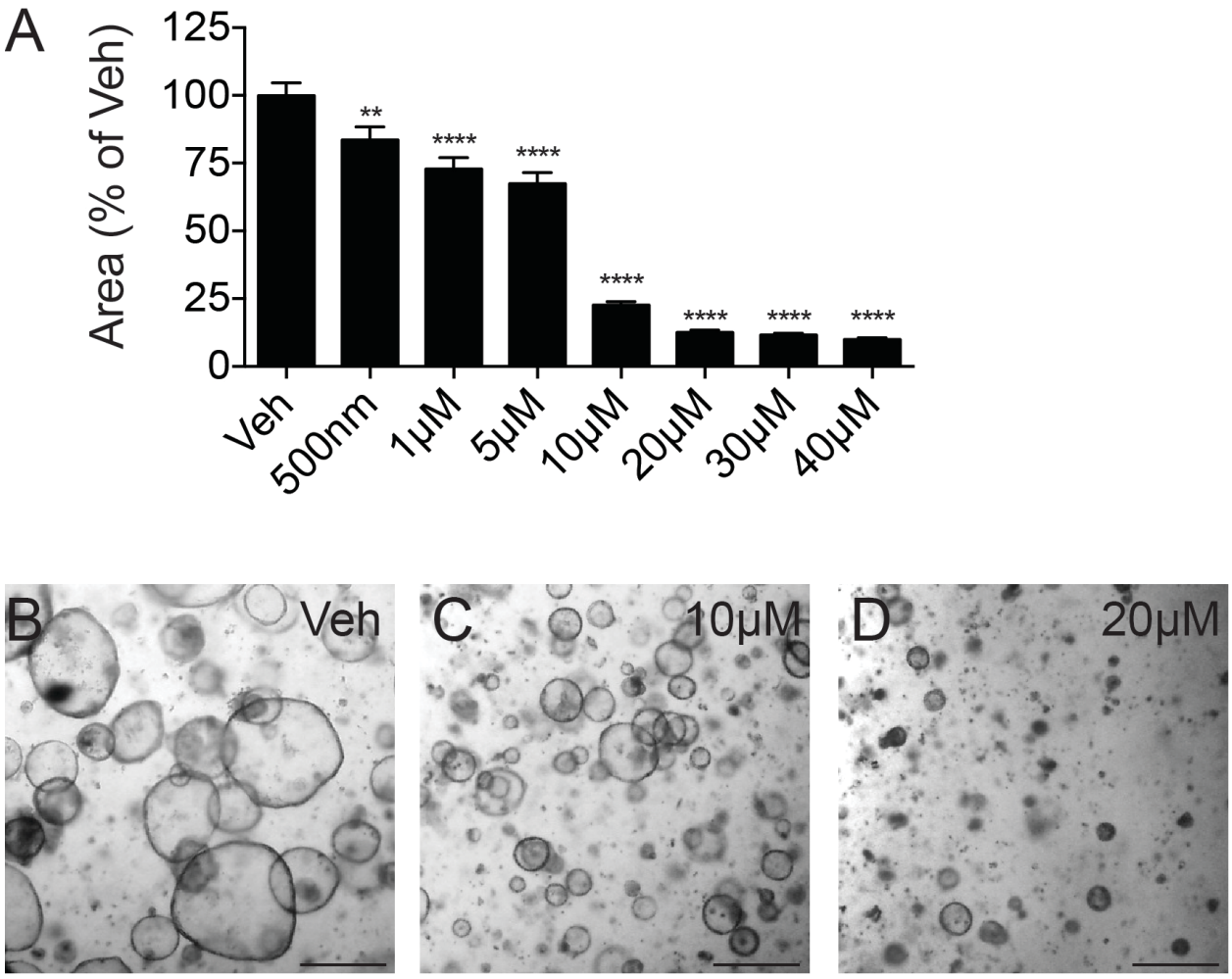

Figure 4-6. Notch inhibition reduces corpus organoid growth in a dose-dependent manner. (A) Corpus organoid growth was measured after 5 days with the addition of varying doses of the γ-secretase inhibitor DAPT (mean ± SEM, n=200 organoids per treatment). Representative images of (B) vehicle, (C) 10μM, or (D) 20μM treated organoids. Scale = 250μm. **P<0.01, ****P<0.0001 vs. Vehicle using 1-way ANOVA.

is shown with 10μM DAPT (Fig 4-6C) compared with vehicle-treated (Fig 4-6B). Significant organoid death is observed with 20μM DAPT (Fig 4-6D).

Human Gastric Organoid Establishment

Tissue for establishment of human gastric organoids was collected from surgical resections, Gift-of-Life, and biopsies. The regional classification of gastric tissue (antrum versus corpus) was determined based on clinical information from physicians and histological characterization by H&E staining (Fig 4-7, Table 4-3). The protocol for isolating gastric glands was the same for biopsies or full-thickness tissue. Briefly, tissue was incubated in DTT followed by EDTA and then shaken to release full glands. Glands were then densely plated in Matrigel and overlaid with media.

Establishment of human gastric organoids utilized 2 different media conditions (see Methods, Table 4-1). Media A involved conditioned media from cell lines secreting Wnt3a[4] and R-spondin2[18] with added supplements and recombinant EGF and Noggin. Large spheroids formed within 1 day of plating glands, but efficiency was very low (<5%). Organoid growth was slow and organoids grew with variable morphology. In contrast to mouse, human organoids cultured with media A had a folded epithelial layer with multiple invaginations rather than a central sphere (Fig 4-8A-C). These organoids persisted for <30 days in culture and did not survive more than 3 passages. Immunohistochemical analysis of organoids showed the invaginated epithelial layer and lack of a central lumen through E-cadherin staining (Fig 4-9A,B). Proliferative cells were restricted to specific regions of the organoid, indicated by clustering of Ki67 immunohistochemistry (Fig 4-9C,D).

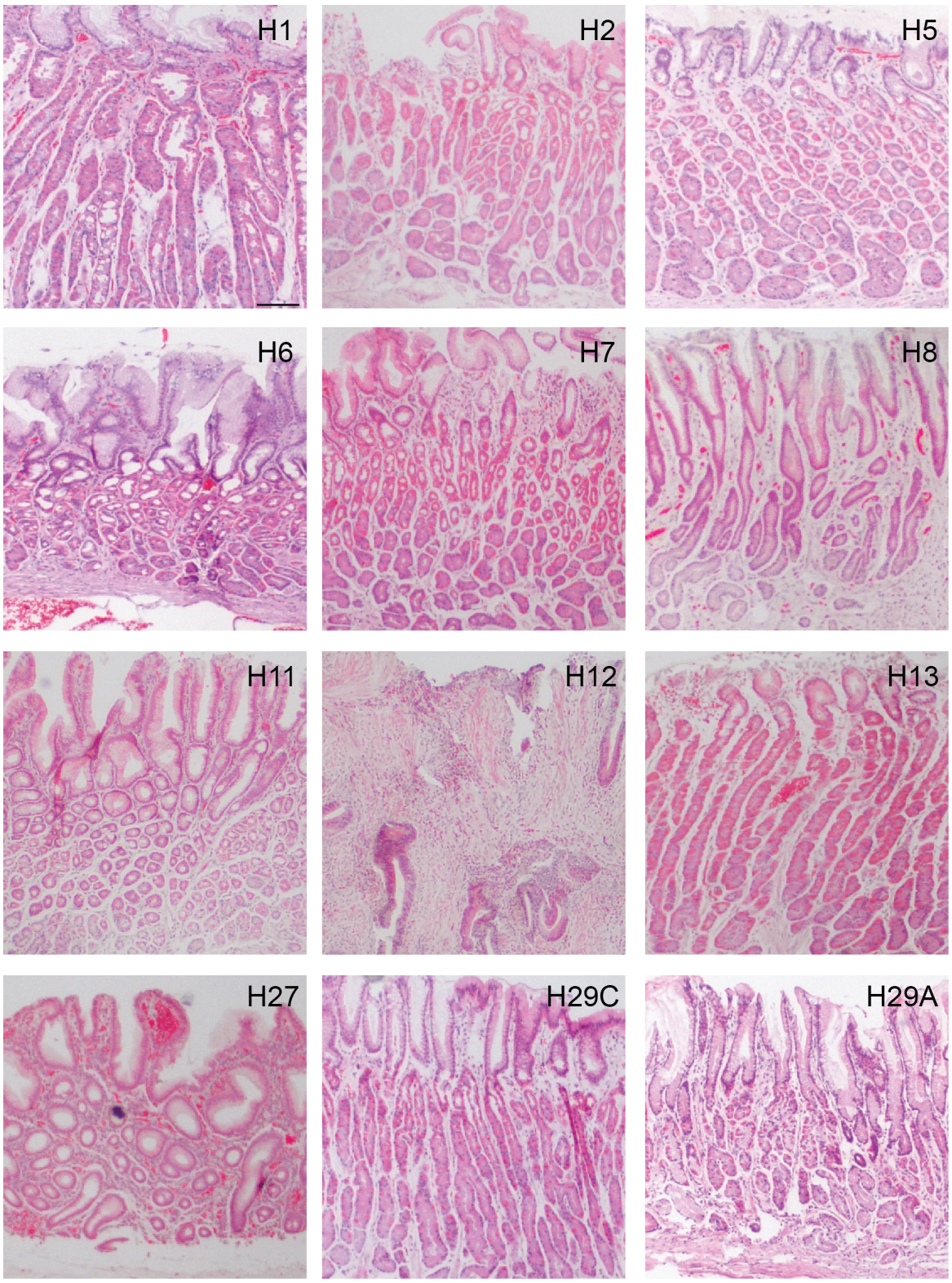

Figure 4-7. Histology of human tissue samples. Paraffin sections of human tissue samples was stained with H&E. Scale = 50μm.

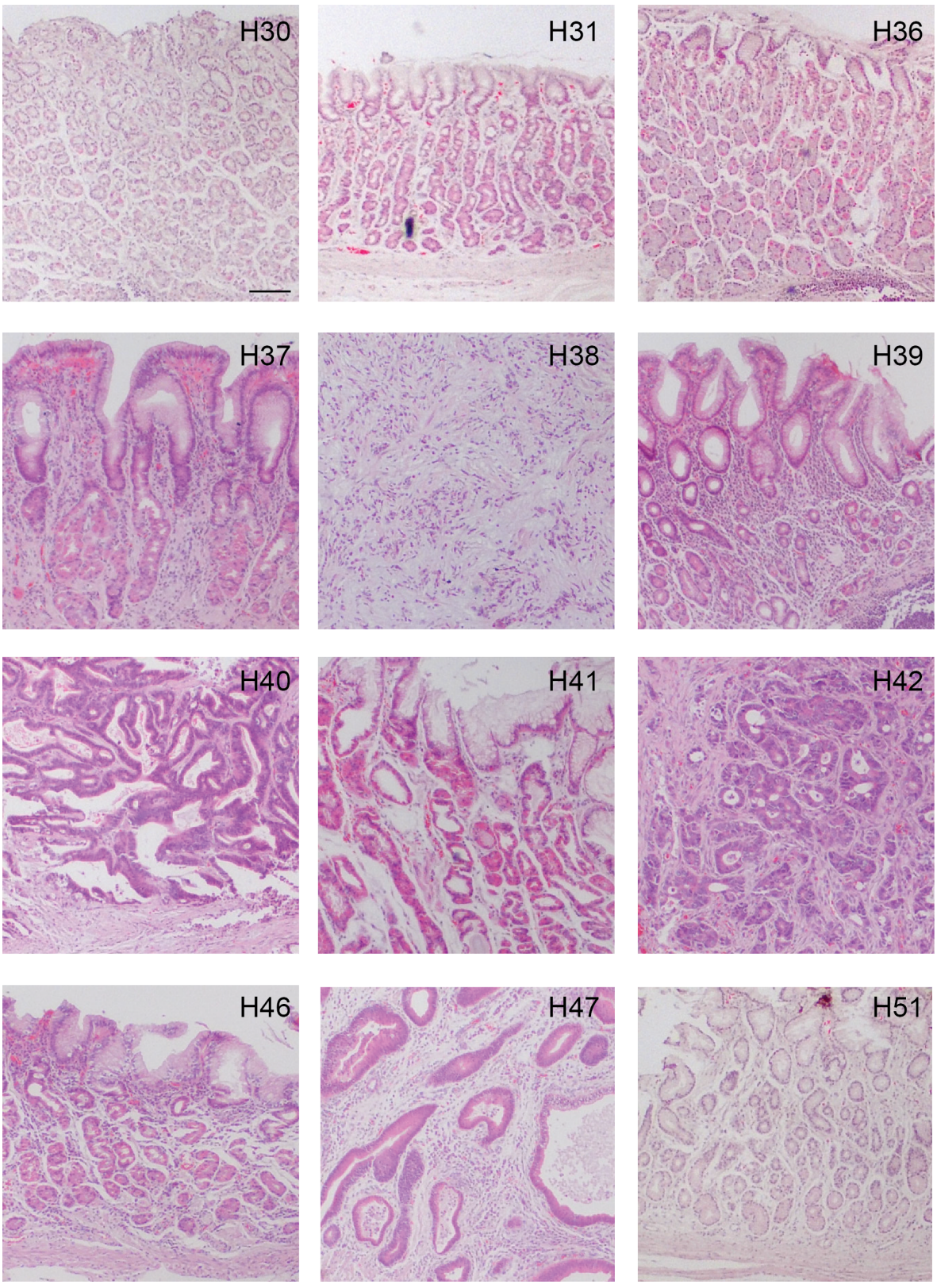

Figure 4-7 cont. Histology of human tissue samples. Paraffin sections of human tissue samples was stained with H&E. Scale = 50μm.

Table 4-3. Characterization of human gastric tissue samples

Patient	Region	Tissue Type
H1	Corpus	Surgical
H2	Antrum	Surgical
H5	Antrum	Surgical
H6	Antrum	Surgical
H7	Antrum	Surgical
H8	Antrum	Surgical
H11	Antrum	Surgical
H12	Gastric tumor (paired to H13)	Surgical
H13	Corpus	Surgical
H27	Antrum	Surgical
H28*	Antrum	Surgical
H29C*	Corpus	Gift of Life
H29A*	Antrum	Gift of Life
H30	Corpus	Gift of Life
H31*	Antrum	Surgical
H36	Antrum	Gift of Life
H37	Antrum	Surgical
H38	Gastric tumor (paired to H37)	Surgical
H39*	Antrum	Surgical
H40	Gastric tumor (paired to H39)	Surgical
H41*	Antrum	Surgical
H42	Gastric tumor (paired to H41)	Surgical
H45*	Corpus	Biopsy
H46*	Antrum	Surgical
H47*	Gastric tumor (paired to H46)	Surgical
H51	Antrum	Gift of Life
H52*	Corpus	Gift of Life

*Organoids were established and expanded from tissue.

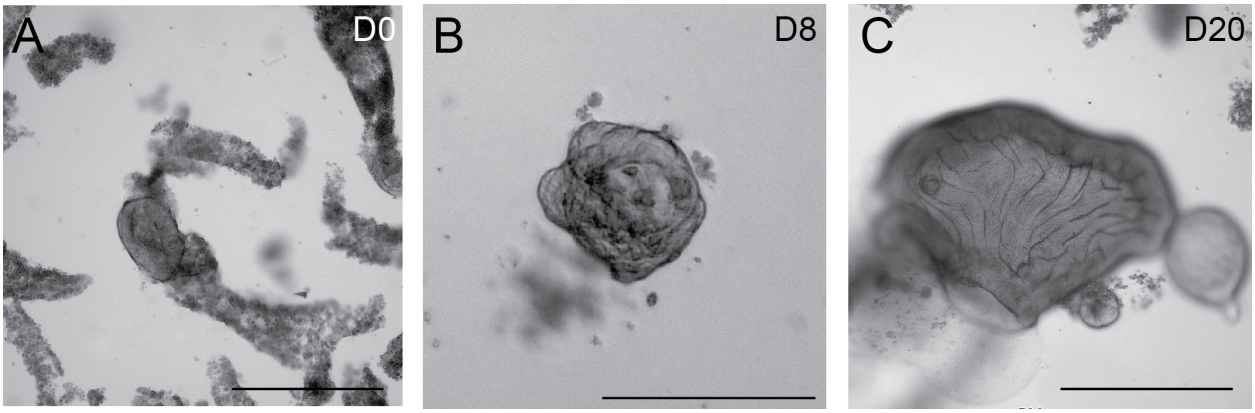

Figure 4-8. Human organoid growth with media A. Organoid growth on day 0 (D0) (A), day 8 (D8) (B), and day 20 (D20) (C) from isolated human antral glands utilizing conditioned Wnt3a and RSPO2 media. Scale = 500μm (A,C), 250μm (B).

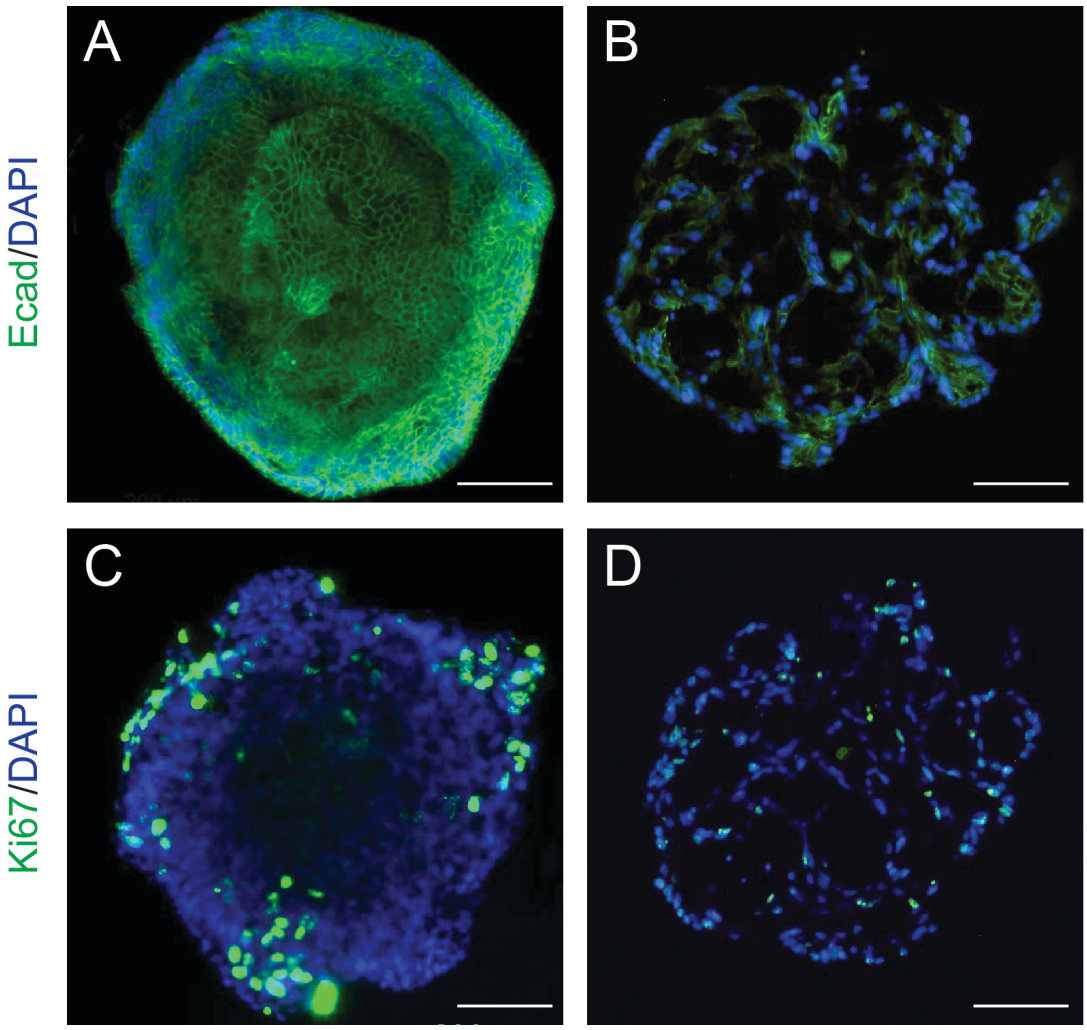

Figure 4-9. Morphology and proliferation of human gastric organoids. Human gastric organoids established with media A exhibit non-spherical morphology, shown with E-cadherin (Ecad) staining (A,B). Human organoids exhibit limited regions of proliferative cells (C,D). Scale = 100μm.

Utilizing media A, organoids were established from gastric polyp tissue (H12). Due to the transformed nature of the tissue, full gland isolation was difficult. Instead, tissue was finely minced after incubation in DTT and EDTA and plated in Matrigel in small pieces. This tissue formed organoids at a much faster rate than was observed after plating glands from normal tissue, with spheroids appearing within 2-3 hours of plating tissue. Polyp organoids also grew extremely rapidly, with large organoids forming within 7 days (Fig 4-10). However, similar to organoids established from normal gastric tissue grown in media A, organoids did not expand with passaging and did not survive more than 2-3 weeks. Though organoids were able to form in this media condition, the low efficiency and lack of expansion proved them to be unusable for further analysis and manipulation.

Media B used conditioned media termed “L-WRN”, which is collected from an L-cell line that produces Wnt3a, R-spondin3, and Noggin[17]. Addition of a ROCK-inhibitor and TGFβ-inhibitor were required for growth. Small spheres formed within 1 day of plating antral glands with higher efficiency compared to media A (>50% of plated glands formed spheroids) (Fig 4-11). Organoids require splitting soon after initial plating (3-4 days) to remove dead cells. All organoids grow as spheres and expand to <250μm, growing best when densely plated (300-400 organoids/40μl Matrigel patty). Organoids expand exponentially when split at a 1:2 ratio and were able to be maintained over 6-months in culture. Similar to the mouse, human organoids established in media B have numerous proliferative cells scattered throughout the organoids (Fig 4-12A) and exhibit limited differentiated cell markers, including mucous cells marked by Griffonia

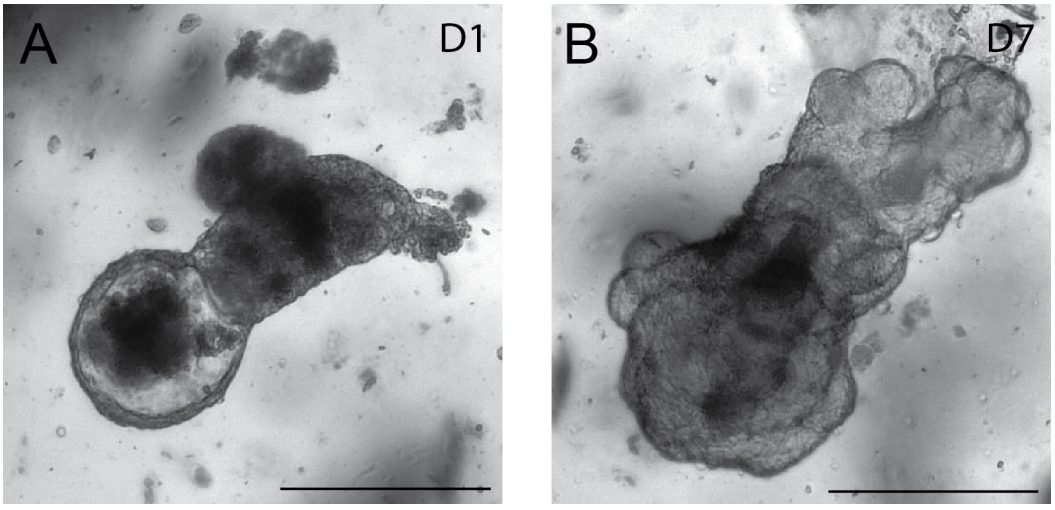

Figure 4-10. Establishment of human gastric polyp organoids using media A. Organoid were established from human gastric polyp tissue (H12) and imaged on Day 1 (D1) (A) and Day 7 (D7) (B). Scale = 250μm.

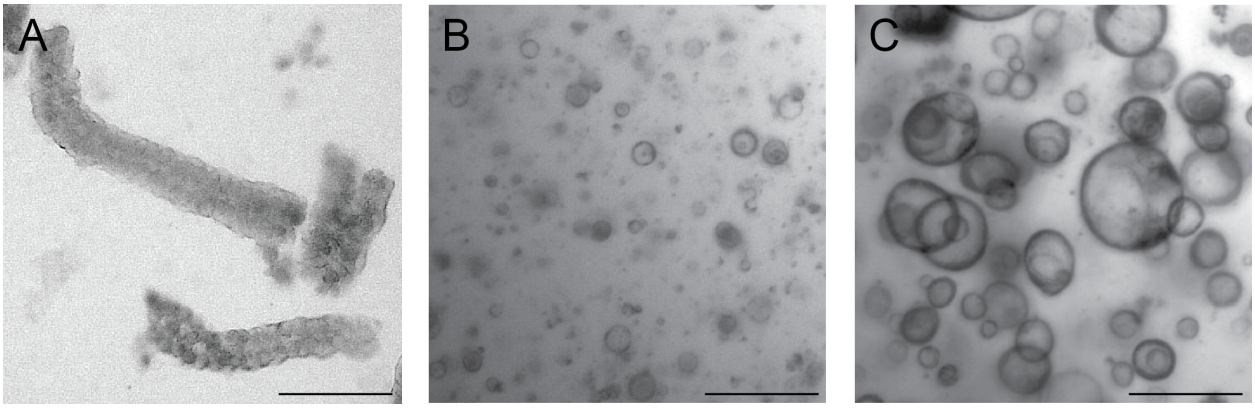

Figure 4-11. Human organoid growth with media B. (A) Isolated human antral glands. Passaged organoids (B) 24 hours after passaging and (C) 5 days after passaging. Scale = 500μm (A) and 250μm (B,C).

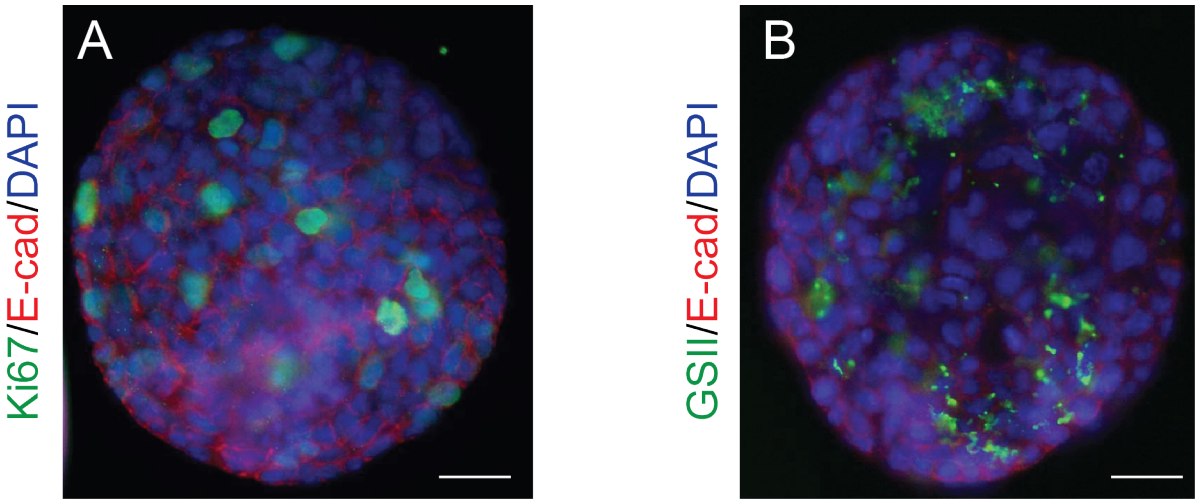

Figure 4-12. Proliferation and differentiation marker staining in human antral organoids. Human antral organoids are composed primarily of proliferating cells (A) marked by Ki67 and exhibit limited differentiated cell types, including (B) mucous cells marked by Griffonia Simplicifolia-II (GSII). Scale = 25μm.

Simplicifolia-II (GSII) (Fig 4-12B). Human antral organoids did not show staining for the G-cell marker gastrin (data not shown). Notch signaling is required for antral and corpus organoid growth, as shown by analysis after DAPT treatment (Chapter 2, Fig 2-4; Chapter 3, Fig 3-5).

Organoids from gastric tumor tissue (H47, Table 4-2) were established with the L-WRN conditioned media. Organoid formation efficiency was lower in tumor tissue compared to normal tissue due to the difficulty of isolating glands from the transformed tissue due to fibrosis. Once established, tumor organoid growth did not differ from normal gastric organoid growth (data not shown).

4.5: DISCUSSION

Organoid culture of human and mouse gastric epithelial cells was used to explore culture conditions to support stem cell growth. By using this culture system, we studied Notch regulation of human and mouse stem cells *in vitro.* I outlined the protocols for culturing human and mouse gastric tissue *in vitro* and characteristics of organoid growth. I also elaborated on how Notch signaling affects mouse and human gastric organoid growth *in vitro.*

Organoids represent a physiologic tissue type that are able to undergo standard experimental manipulations that have been used for cell lines, including long-term storage in liquid nitrogen, infection with recombinant viruses, and transfection of DNA and siRNA[20]. They can be analyzed by immunohistochemistry and gene and protein expression. Organoids can be genetically manipulated by establishing from mice harboring the Cre/loxp system and treating with tamoxifen *in vitro,* or treated with small

molecules to inhibit gene or protein functions[21]. Alternatively wild-type organoids can be genetically manipulated *in vitro* by infection with Cre recombinase-inducible retrovirus vectors that allows for the conditional manipulation of gene expression in organoids with loxP targets[22].

Organoids as a regenerative stem cell therapy shows significant promise. In one study, organoids were established from the colon of EGFP transgenic donor mice. After expansion, organoids were fragmented and transplanted by enema into mice with colonic mucosal damage by dextran sulfate sodium (DSS) treatment. The organoids engrafted into the mucosal lesions and were indiscernible from surrounding recipient epithelium for at least 6 months and exhibited all normal cellular proliferative and differentiated cell types in appropriate locations[23]. This study provides proof of principle that cultured organoids can be used to repair damaged epithelium. The future goal of organoids as a source of regenerative tissue in humans is appealing due to lack of common complications typically associated with tissue transplant due to graft-versus-host diseases. Ideally a person's own epithelial cells could be isolated and cultured to be re-inserted to treat pathologies such as ulcers. However, complications that must be considered include maintaining an immaculate genome as well as developing artificial 3D matrices that are free of growth factors[20].

Organoids can be established from both normal and cancerous tissue[3]. A recent study established tumor organoid cultures from 20 colorectal carcinoma patients to establish a "living biobank". Because organoids can be rapidly expanded, each line underwent gene expression analyses and was subjected to high-throughout drug screens. This allowed for detection of gene-drug associations and generated

reproducible data that detected sensitivity of the tumor tissue to specific drug combinations[24]. Because of the inherent complexity of cancer development at the gene level, studies like this with the “living biobank” can identify complex interactions between multiple genomic alterations to help predict drug sensitivity outcomes. This technology could be translated to the concept of personalized medicine, in which an individual’s own gastrointestinal epithelial cells can be used to screen for novel therapies and targeted treatment for their specific pathology. For further study of gastric cancer development, biobanks of gastric organoids could be established and studied to identify specific gene mutations similar to what has been done with colon organoids. Organoids may be the link between cancer genetics and patient trials in allowing personalized therapy design.

The organoid system has fueled “a renaissance of *in vitro* studies of human intestinal development and disease”[25]. The power of this method has opened up new avenues in the exploration of human gastrointestinal physiology in understanding the basic biology of normal epithelial homeostasis. Organoids will serve as a novel tool in personalized medicine and furthering cancer and other disease research by establishment of biobanks for identification of common gene mutations and preliminary therapeutic testing.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The research described for this thesis has advanced the field of gastric stem and epithelial cell biology by furthering our understanding of how the Notch signaling pathway regulates gastric epithelial cell dynamics. I have addressed this question by observing the effects of pharmacological inhibition of the NOTCH1 and/or NOTCH2 receptors *in vivo* and *in vitro* to deduce the individual role of these receptors on maintaining homeostasis in the gastric epithelium.

In this chapter I will discuss the findings of my research and how it furthers the field. I will also identify some questions that my work raises and present some preliminary data and discuss experiments that can address these questions.

NOTCH1 and NOTCH2 regulate gastric antral epithelial cell proliferation

In Chapter 2, I discovered a role for NOTCH1 and NOTCH2 in the regulation of antral epithelial cell homeostasis. I first characterized Notch receptor expression in mouse antral tissue by utilizing transgenic reporter mice to identify cells undergoing active Notch signaling as well as cells expressing NOTCH1 or NOTCH2. Active Notch signaling was present in both non-epithelial and epithelial cells, including

enteroendocrine, tuft, and stem cells. NOTCH1 expression was seen in antral stem cells as evident from lineage tracing driven by the Notch1-CreERT2 promoter resulting in full antral-gland tracing. NOTCH2 did not appear to be expressed in stem cells, as no full antral-gland lineage tracing was observed in mice 2 weeks after tamoxifen activation. However, analysis of tissue 3 days after tamoxifen activation showed NOTCH2-expressing epithelial cells are in the proliferative zone of antral glands. These findings suggest that while NOTCH1 is expressed in antral stem cells, NOTCH2 is not present in stem cells, but instead is expressed in progenitor cells. *Notch1* and *Notch2* expression was previously identified in neonatal mice[1], and my work furthers our understanding of Notch in the adult mouse stomach by identifying which receptors are expressed and the cellular identity of receptor-expressing cells in the antrum.

Further characterization of the role of NOTCH1 and NOTCH2 in antral epithelial homeostasis revealed a reduction in proliferation with inhibition of both receptors, with a milder reduction seen with individual receptor inhibition. Reduction of proliferation from global inhibition of the Notch signaling pathway has been previously reported[1,2]. However, the Notch receptor specificity was not known. I showed that inhibition of NOTCH1 and NOTCH2 mimicked the reduction in proliferation seen with global Notch inhibition, indicating that these two receptors are key in gastric Notch signaling.

NOTCH1 and NOTCH2 regulate antral epithelial cell differentiation

Notch signaling was previously identified as a regulator of gastric cell differentiation, but receptor specificity was unknown[1,2]. Analysis of changes to cellular differentiation with inhibition of Notch receptors revealed that inhibition of both NOTCH1

and NOTCH2 resulted in an increase in enteroendocrine, deep mucous, and surface mucous cells. These cellular changes did not occur with inhibition of NOTCH1 or NOTCH2 alone. Thus, this increase in differentiated cells types mimicked the increase seen with global Notch inhibition, indicating that together these two receptors are key to maintaining proper epithelial differentiation.

One unexpected finding from my studies was the observation that Notch inhibition resulted in remodeling of cells in the antral gland base. I observed co-staining for the deep mucous cell marker TFF2 with a marker normally associated with cells in the corpus region (GIF). Moreover, ultrastructure analysis showed an increased density of secretory granules at the base and surface of the antral glands. Increased expression of GIF was also observed with Notch inhibition of antral organoids, demonstrating that the cellular remodeling response is due to direct epithelial cell responses. Further analysis identified increased expression of numerous intestinal and corpus secretory cell markers, indicating a change in rostrocaudal antral-specific gene expression. This was not a remodeling of the antrum into the corpus or intestine, as only a subset of markers are observed while antral markers are maintained. The changes in gene expression suggest the differentiation program is altered and further gene expression analysis is warranted to identify transcription factors that may be altered with Notch inhibition to produce this non-regional gene expression. TFF2-GIF co-stained cells have been previously observed in the newborn stomach[3]. It would be interesting to further investigate how these remodeled cells in the adult Notch-inhibited antrum related to the previously observed occurrences of the co-stained cell types.

Mouse and human antral organoids are regulated by Notch signaling

The organoid culture system allows for the investigation of epithelial-specific effects of Notch inhibition on growth and differentiation of cells. Non-epithelial Notch receptor expression was observed *in vivo*, so organoids were a beneficial tool to determine if Notch regulation of gastric homeostasis is intrinsic to the epithelium. Treatment of both mouse and human gastric organoids with Notch inhibitors resulted in a similar reduction in organoid growth with global, NOTCH1 + NOTCH2, and NOTCH1 inhibition, with a less significant growth reduction seen with Notch2 inhibition. This is surprising due to the observation that NOTCH1 and NOTCH2 inhibition alone resulted in a similar level of reduced proliferation that was intermediate to global or NOTCH1 + NOTCH2 inhibition *in vivo,* thus I expected to see comparable growth inhibition *in vitro*. The epithelial-specific culture identified Notch1 as playing a larger role in the regulation of proliferation in the epithelium compared to NOTCH2. Hypotheses as to why I see a larger role of NOTCH1 as compared to NOTCH2 on organoid growth *in vitro* are described in the next section.

The finding that Notch regulates human organoid growth was a significant advancement in the understanding of human gastric stem cell biology. Previous analysis of signaling pathways regulating human gastric epithelial homeostasis was based on gene expression studies comparing normal versus diseased tissue or utilizing human gastric cancer cell lines[4–6]. Utilization of the human gastric organoid system allows for direct manipulation of normal cultured human gastric stem cells and observation of gene or protein changes over time. I am the first to identify Notch signaling as a key regulator of normal human gastric stem cell homeostasis *in vitro.* My

findings also validated the use of mouse models for the study of gastric physiology, in that both the mouse and human organoids responded similarly to specific Notch receptor inhibition.

Different function of antral NOTCH2 in vivo and in vitro

Significant Notch expression was present in non-epithelial cells, primarily localizing to endothelial and smooth muscle cells. I observed a difference in the role of NOTCH1 compared to NOTCH1 *in vivo* versus *in vitro,* with NOTCH1 playing a larger role in regulating epithelial proliferation *in vitro.* There are two potential hypotheses as to why this difference is occurring *in vitro* compared to *in vivo.*

One can hypothesize that non-epithelial Notch signaling is playing a role in the regulation of epithelial proliferation *in vivo*. The non-epithelial cells that showed Notch signaling included cells in the proposed stem cell niche at the base of antral glands that co-stained with PECAM, a marker of endothelial cells. Utilization of an endothelial cell-specific Cre[7–9] for genetic deletion of Notch components, such as *Rbpj* or Notch receptors, would allow for testing whether Notch component expressing cell type contributes to epithelial homeostasis. One study showed a gastrointestinal effect of *Rbpj* deletion in endothelial cells[10]. Analysis of neonatal mice with an endothelial-specific Cre driving deletion of *Rbpj* resulted in expanded vascular lumens in mesentery and submucosa in the intestine[10], but no data was shown as to epithelial cell proliferation or differentiation. To test the role of endothelial Notch receptors on epithelial homeostasis, one could utilize the inducible endothelial-specific VE-cadherin-CreERT2[9] mouse strain crossed with either Notch1$^{floxed/floxed}$ or Notch2$^{floxed/floxed}$ mice (Jackson Lab no. 007181

and 010525) and activate receptor gene deletion in adult mice with tamoxifen treatment. One can test effects on epithelial proliferation; hypothesizing that if Notch expression in non-epithelial cells influences epithelial cell homeostasis, one would expect to see decreased proliferation with deletion of Notch components in endothelial cells. Furthermore, endothelial cells can be harvested from the stomach and establish organoids on a plate seeded with Notch1 and/or Notch2-deleted endothelial cells to see if there is a growth advantage or impairment on organoids[11].

An alternative hypothesis for why the Notch receptors have a differential effect on organoid growth could be due to the cellular make-up of organoids. In my culture conditions, organoids are primarily composed of proliferative cells. From *Notch1-Cre* and *Notch2-Cre* lineage tracing I see that *Notch1* expression is present in gastric stem cells. The organoids might show a greater reduction in growth with NOTCH1 inhibition due to the fact that there are more *Notch1*-expressing cells present in the organoids that are regulating the overall growth compared to *Notch2*-expressing cells. To test this hypothesis, I could alter the culture conditions of the organoids to promote cellular maturation and a reduction in proliferation by lowering the amount of Wnt and R-spondin in the media. I could also co-culture organoids with mesenchymal cells in a protocol devised by the Zavros lab that promotes maturation of differentiated lineages and a reduction in proliferating cells[11] to better mimic the *in vivo* cellular composition.

NOTCH1 and NOTCH2 regulate corpus epithelial proliferation

In chapter 3, I tested the role of NOTCH1 and NOTCH2 in the corpus. I characterized the mRNA expression levels of the Notch receptors in the corpus and

identified *Notch1* and *Notch2* as the primary receptor transcripts. Global inhibition of Notch signaling or specific blocking of Notch1 and/or Notch2 reduced proliferation. Similar to the antrum, proliferation was reduced to comparable levels with either global Notch inhibition or NOTCH1 and NOTCH2 inhibition, with single NOTCH1 or NOTCH2 inhibition leading to an intermediate reduction in epithelial proliferation. This finding expands what had been previously published showing a reduction of proliferation with global Notch inhibition[1] by identifying the specific Notch receptors that are responsible for regulation of corpus epithelial proliferation.

I also derived organoids from mouse and human corpus tissue to investigate if Notch signaling was regulating corpus proliferation in an epithelial-specific manner. Both mouse and human corpus organoids show a reduction in growth with global Notch inhibition and with NOTCH1 and NOTCH2 inhibition that mimics the reduction in proliferation seen *in vivo*. Inhibition of NOTCH1 or NOTCH2 resulted in an intermediate reduction in growth, similar to what is seen *in vivo.* This indicates that Notch is acting in an epithelial-specific manner for the regulation of proliferation *in vivo* and *in vitro.*

Lineage tracing experiments from *Notch1-Cre* and *Notch2-Cre* mice did not show any lineage tracing in the corpus epithelium after a 2-week chase period following Cre activation. Instead, *Notch1* and *Notch2* were expressed in single enteroendocrine, chief, and parietal cells. The lack of lineage tracing has 2 potential explanations, 1) NOTCH1 and NOTCH2 are not expressed in stem/progenitor cells of the corpus or 2) the epithelial turnover rate is longer than the 2-week timepoint, leading to only singly labeled cells instead of tracing. However, based on the shorter turnover rate for surface mucous cells we expected to see tracing to this cell type if these Notch receptors were

expressed in a stem or progenitor cell, and further investigation as why we did not observe this is necessary. Due to effects on epithelial cell proliferation, we hypothesize that NOTCH1 and NOTCH2 regulate stem or transit amplifying cells. Longer timepoints may need to be analyzed to fully investigate the localization of NOTCH1 and NOTCH2 in corpus stem cells. The turnover rate of chief cells is the longest of the corpus cell types, at over 6 months[12], so a 6-month chase period after Cre activation would be necessary for thorough examination.

Limitations to observing changes to differentiated cells in the corpus with Notch inhibition

With Notch inhibition of proliferating cells in the mouse corpus, I expected to see an increase in differentiated cells types, as was observed in the antrum. However, analysis of chief, parietal, enteroendocrine, deep mucous, and surface mucous cells did not show any change in marker expression with global or receptor-specific Notch inhibition. One explanation for the lack of changes is the extended turnover time required for differentiated cells of the corpus compared to the antrum. The turnover time for corpus cells can be as high as 6-months for chief cells[13], however we are limited to a timepoint of 6 days with global or Notch1 and Notch2 receptor inhibition due to animal morbidity resulting from intestinal toxicity[14,15]. Unfortunately, we do not currently have a genetic Cre driver that is expressed exclusively in actively cycling corpus stem and/or epithelial cells to avoid this toxicity and allow for longer timepoints. Current gastric stem cell markers include *Lrig*[16], which marks active stem cells in the stomach but also the intestine, *Troy*[17], which marks quiescent stem cells, and *Tff2*[18], which marks the

progenitor for chief and parietal cells only. Preliminary studies in our lab extending the original finding of *Sox2* as a gastric stem cell marker[19] have verified *Sox2* as a potential Cre driver for stomach but not intestinal recombination, and further analysis crossing it to *Notch1*$^{floxed/floxed}$ or *Notch2*$^{floxed/floxed}$ mice (Jackson Lab no. 007181 and 010525) will allow us to investigate the loss of *Notch1* and/or *Notch2* in the corpus for prolonged timepoints.

Mouse and human gastric organoid culture

In chapter 4 I summarized the conditions I optimized for growth of human and mouse gastric organoids. Using culture conditions that include Noggin, Wnt, and R-spondin as well as additional growth factors and small molecules, such as a ROCK-inhibitor and TGFβ-inhibitor, I successfully established organoids and showed they can be expanded for over 6-months in culture. The organoid cultures were primarily composed of proliferating cells. They did express markers of differentiated cells, shown through immunohistochemistry and gene expression analysis. Mouse and human organoid growth was sensitive to Notch signaling, with a reduction in overall growth with inhibition of the Notch signaling pathway.

One limitation of the culture conditions that I optimized is the lack of robust differentiated cell gene expression. Because my primary interest was to study Notch effects on proliferation this culture system worked well. The culture conditions could be modified to induce more differentiated cell types and reduce proliferating cells, similar to what has been published from other labs[20,21], such as reducing the amount of Wnt or R-spondin supplied in the media. Also, the Zavros lab published conditions utilizing co-

culture of gastric organoids with mesenchymal cells, which resulted in an increase in differentiated cell types and decrease in proliferating cells[11]. It is hypothesized that these mesenchymal cells secrete factors required for *in vitro* differentiation that are not being supplied by the media. This co-culture can be used to further analyze changes to differentiated cell types in antrum and corpus organoids after Notch inhibition, and can potentially be applied to human corpus and antrum organoids to create a more physiologically relevant culture system to study mechanisms of cellular differentiation.

The role of Notch ligands in gastric epithelial cell homeostasis

This thesis analyzed the role of the Notch receptors in the regulation of gastric epithelial cell homeostasis. In order to fully understand the mechanism with which Notch regulates the gastric epithelium, the role of specific Notch ligands and their contribution to the gastric stem cell niche needs to be described. In the intestine, a comparison of genetic deletion of *Dll1*, *Dll4*, and *Jag1* indicated that *Dll1* and *Dll4* are the primary ligands regulating intestinal stem cell homeostasis[22]. *Dll1* deletion resulted in a moderate secretory cell hyperplasia, but deletion of both *Dll1* and *Dll4* mimicked *Rbpj* deletion, indicating Notch signaling is regulating intestinal stem cell homeostasis through these ligands[22]. *In situ* hybridization showed that *Dll1* and *Dll4* are expressed in cells localized to the base of the crypts, presumably next to intestinal stem cells, indicating a role in the intestinal stem cell niche [23]. However, the intestinal cell that expresses Notch ligands is not clear, with TA, Paneth and enteroendocrine cells being present in the intestinal stem cell region. The requirement that Notch signaling occurs

between adjacent cells suggests that either Paneth cells and/or TA cells might be the most likely source of Notch ligand.

In the stomach, the only reports of Notch ligands come from correlation of gene expression in gastric cancer tissue and cell lines[24,25]. I have done an analysis of expression of Notch ligands in mouse and human gastric tissue (Fig 5-1). My studies show that *Jag1* and *Dll1* are the most highly expressed ligands in both mouse and human gastric tissues. In the human gastric tissue, *Jag1* is the most predominant Notch ligand that is expressed. In the mouse gastric tissue, both *Jag1* and *Dll1* are highly expressed at similar levels, with an appreciable expression of both *Jag2* and *Dll4*. This, however, does not mean that the most highly expressed ligands are the ones functioning in the epithelium due to non-epithelial ligand expression.

Detailed analysis is needed to both localize Notch ligands to specific epithelial cells and identify the function of specific ligands. The Watanabe lab published immunohistochemical staining for DLL1 and DLL4 in the base of intestinal crypts using commercially available antibodies, which could be used to identify the localization of these ligands in the stomach[23]. The functional analysis of the role of the Notch ligands can use pharmacological or genetic tools. Inhibitory antibodies specific for DLL1 and DLL4[26] and JAG1[27] have been described and can be used to inhibit the Notch ligands both *in vivo* and *in vitro*. Treating mice or organoids with specific ligand inhibitory antibodies will test which ligands lead to proliferation and differentiation effects similar to what I have shown with receptor inhibition. A genetic approach could also be used, as floxed *Dll1*[22], *Dll4*[22], *Jag1*[28] (Jackson Labs no. 010618), and *Jag2*[29] mice have been described. These mice can be crossed to specific Cre drivers to delete genes in the

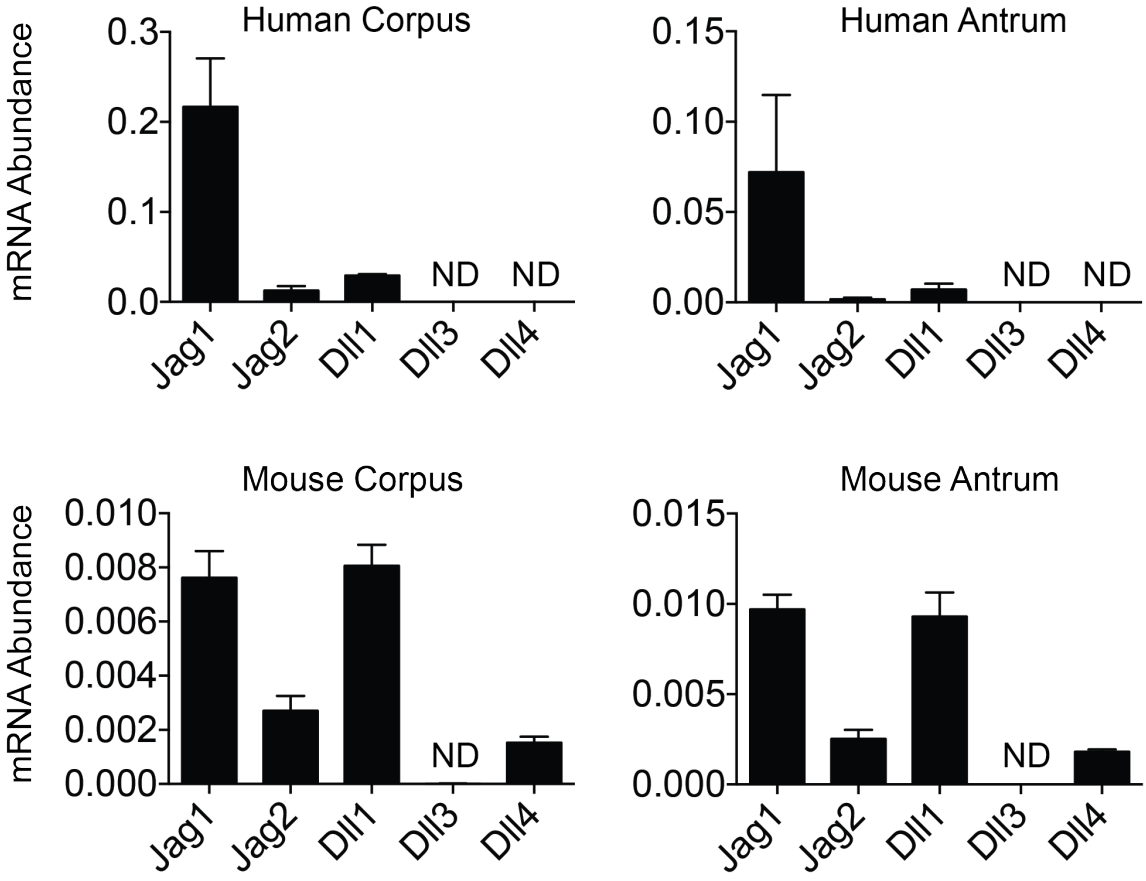

Figure 5-1. Expression of Notch ligands in mouse and human gastric tissue. Gene expression of Notch ligands from human and mouse corpus and antral tissue was determined by qRT-PCR (mean ± SEM, n=3). ND = Not Detected.

stomach, ideally with a stomach-specific Cre driver such as *Sox2*[19] to avoid intestinal effects.

Comparison of the role of NOTCH1 and NOTCH2 in the stomach and intestine

The role of the Notch receptors in regulating intestinal stem cell homeostasis has been well characterized[14,15,26,30,31]. Global Notch inhibition in the intestine led to a reduction in proliferation and secretory cell hyperplasia, which was also seen with inhibition of both NOTCH1 and NOTCH2[14,26,30]. Inhibition of NOTCH1 alone was sufficient to induce some goblet cell metaplasia, but the effect was mild compared to NOTCH1 and NOTCH2 inhibition[26,30]. NOTCH2 inhibition alone did not produce any phenotype under homeostatic conditions[26,30].

My findings in the stomach also identified NOTCH1 and NOTCH2 as the key receptors in regulation of gastric epithelial differentiation in the antrum and proliferation in both glandular regions. However, I saw a partial phenotype of reduced proliferation with inhibition of NOTCH1 or NOTCH2 alone, which was not observed in the intestine. I did not see any phenotype in differentiated cells with NOTCH1 or NOTCH2 inhibition, indicating that these receptors function redundantly in antral cell differentiation.

With NOTCH1 and NOTCH2 being key for regulation of both gastric and intestinal epithelial cell homeostasis, it is important to appreciate changes to both regions with inhibition of the receptors. Due to intestinal toxicity with global Notch or NOTCH1 and NOTCH2 inhibition, the receptors may have limitations as therapeutics targets for gastric pathologies. However, investigation of the Notch ligands in the stomach is intriguing due to the high expression of *Jag1*, which is not involved in

intestinal stem cell maintenance[22]. Future studies are needed to identify its function for gastric epithelial cell homeostasis.

In conclusion my studies have shown that the Notch signaling pathway is important for the regulation of mouse and human gastric epithelial homeostasis. My thesis work has illustrated an important role for the NOTCH1 and NOTCH2 receptors in regulating gastric epithelial proliferation and differentiation both *in vivo* and *in vitro*. Additionally, I have described the novel finding of Notch regulation of human gastric corpus and antral cell homeostasis *in vitro,* which is the first analysis of the regulation of human gastric stem cells by Notch. There are many remaining questions as to the mechanism with which Notch regulates gastric stem cells and how it can be misregulated to lead to pathologies, such as gastric cancer.

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