NOTCH1 AND NOTCH2 RECEPTORS REGULATE HUMAN AND MOUSE GASTRIC EPITHELIAL CELL HOMEOSTASIS

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Molecular and Integrative Physiology) in The University of Michigan 2016

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This thesis is dedicated to my family: Mom, Dad, Katie, and Dan. For your continuous love, support, and encouragement.
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ABSTRACT

NOTCH1 AND NOTCH2 RECEPTORS REGULATE HUMAN AND MOUSE GASTRIC EPITHELIAL CELL HOMEOSTASIS

by

Gail B. Gifford

Chair: Linda C. Samuelson

The gastric epithelium undergoes constant turnover that is maintained by a population of gastric stem cells. Gastric stem cells are under the regulation of multiple signaling pathways to promote proper epithelial homeostasis. Previous studies have shown that the Notch signaling pathway plays a crucial role in regulating epithelial differentiated cell fate, stem cell function, and epithelial cell proliferation in the stomach. My thesis work has focused on identifying the mechanisms by which Notch signaling regulates gastric epithelial cell homeostasis.

I identified Notch1 and Notch2 as the key receptors contributing to the regulation of gastric epithelial cells by Notch. Using inhibitory antibodies targeting Notch1 and Notch2, I observed a marked reduction in proliferation of both corpus and antral epithelial cells that mimicked the reduced proliferation observed with global Notch inhibition. Inhibition of Notch1 or Notch2 signaling led to an intermediate reduction in
proliferation in both regions of the glandular stomach. In the antrum, inhibition of both receptors resulted in a general increase in expression of markers of differentiated cells, including enteroendocrine, surface mucous, and deep mucous cells. Inhibition of both receptors also led to increased secretory granules in antral cells and expression of secretory products from other regions of the gastrointestinal tract, including the corpus and intestine.

To investigate if Notch signaling is intrinsic to the epithelium, I refined the conditions for gastric organoid establishment from mouse and human antrum and corpus tissue. In mouse and human antral and corpus organoids, inhibition of Notch1 and Notch2 resulted in a reduction of organoid growth similar to that seen with global Notch inhibition. In corpus organoids, inhibition of either Notch1 or Notch2 resulted in an intermediate disruption of organoid growth. However, in antral organoids, inhibition of Notch1 mimicked growth similar to that seen with global inhibition, suggesting that Notch1 may play a more significant role in antral organoid growth than Notch2.

In summary, my thesis work has expanded the understanding of the role of Notch in gastric epithelial homeostasis. I have illustrated an important role for the Notch1 and Notch2 receptors in regulating gastric epithelial proliferation and differentiation in vivo and in vitro.
CHAPTER 1

INTRODUCTION

Stem cells are undifferentiated cells capable of self-renewal and division into multiple differentiated cell types. Stem cells are key cells to maintain tissue homeostasis in all multicellular organisms. From initial development from a single pluripotent embryonic stem cell through adult organ-specific maintenance by tissue-specific stem cells, an organism’s viability depends on the proper functioning of these stem cells. The balance of proliferation and differentiation is key to not only maintaining proper function, but also to prevent unchecked stem cell proliferation that may contribute to disease.

The epithelium of the adult stomach is maintained through the proper functioning of stem cells, which proliferate and differentiate into all glandular cell types. The homeostasis of these stem cells is crucial to produce adequate numbers of cells to maintain epithelial integrity. Unchecked proliferation of gastric stem cells can contribute to hyper-proliferative states, such as gastric cancer. Understanding how these gastric stem cells function not only contributes to the advancement of stem cell physiology, but also provides a mechanism of how aberrant stem cell activity can contribute to disease development.
A key question in gastric stem cell biology is how signaling pathways play a role in maintaining stem cell homeostasis. The Notch signaling pathway is a conserved cell-cell signaling pathway that is crucial for maintenance of skin, hematopoietic, intestinal, and other tissue-specific stem cells\(^1\). In the intestine, Notch is a regulator of stem and proliferative cell maintenance and directs cell fate decisions between absorptive versus secretory cell types\(^2\text{--}^7\). The components of the Notch pathway involved in intestinal regulation have been uncovered, with the Notch receptors Notch1 and Notch2 and Notch ligands Delta-like1 (Dll1) and Delta-like 4 (Dll4) maintaining intestinal epithelial homeostasis\(^5,6,8,9\). Notch is also a key regulator of gastric stem and epithelial cell homeostasis\(^10,11\). However, the Notch components regulating gastric stem and epithelial cells have not been uncovered. The goal of this thesis is to investigate the mechanism of Notch regulation of gastric epithelial cell homeostasis. Using transgenic mouse models, pharmacologic inhibition, and \textit{in vitro} organoid culture, my studies addressed the role of the Notch receptors in regulating gastric corpus and antral epithelial cell homeostasis in mouse and human.

This chapter covers three broad topics: (1.1) an overview of gastric stem and epithelial cells and signaling pathways involved in maintaining homeostasis, (1.2) the Notch signaling pathway, and (1.3) the current understanding of the role of Notch signaling on gastrointestinal stem and epithelial cell maintenance. Finally, this chapter concludes with an overview of the experimental approaches and main findings of the thesis (1.4).
1.1: GASTRIC STEM AND EPITHELIAL CELLS

Anatomy and compartments

The stomach is a muscular organ located in the gastrointestinal tract that is important for proper digestion. The mouse stomach can be divided into three parts: the forestomach and the glandular corpus and antrum. The glandular stomach regions have distinct functions. The corpus is responsible for acid secretion while the antrum is responsible for mucous and hormone secretion. A transition zone exists between the corpus and antrum and contains features of both regions. The simple columnar glandular epithelium is organized into multiple gastric units, or glands, which are invaginations of epithelial glands into the underlying mesenchymal tissue. The epithelium of the stomach is highly dynamic with continuous proliferation, migration, differentiation, and apoptosis. Along with different functions, the architecture and epithelial cell types differ in the corpus and antrum.

Corpus gland structure and cell types of the mouse stomach

The corpus is composed of long tubular glands that open into the stomach lumen (Fig 1-1). Corpus glands can be divided into four regions: the pit, isthmus, neck, and base. Stem cells located in the isthmus divide to produce new stem cells or transit amplifying (TA) cells. TA cells are undifferentiated cells that divide more frequently than stem cells to increase the overall cell population in a gastric gland. TA cells include preparietal, prepit, or preneck cells\textsuperscript{12-14}. These TA cells are able to migrate
Figure 1-1. Mouse corpus gland. (A) Histological section of mouse corpus gland stained with haematoxilin and eosin. (B) Cartoon depiction of mouse corpus gland with various cell types identified with specific markers used in this study to identify the differentiated cell types. Mucin 5AC (MUC5AC), chloride channel accessory 1 (CLCA1), trefoil factor 2 (TFF2), griffonia simplicifolia II (GSII), chromogranin A (CHGA), H/K ATPase α subunit (ATP4A), gastrin intrinsic factor (GIF). Scale = 25 µm.
bidirectionally to differentiate and populate all four regions of the corpus gland (Fig 1-1, 1-2).

Surface mucous cells secrete mucous and are primarily located in the pit segment of the corpus gland. Surface mucous cells contain densely packed mucous granules and express mucin 5AC (MUC5AC), gastrokine-1, and trefoil factor family 1 (TFF1); they are generated from prepit progenitor cells\textsuperscript{12,15}. The Hedgehog pathway may play a role in surface mucous cell generation, with a loss of surface mucous cells in primary mouse gastric epithelial cultures treated with cyclopamine, a Hedgehog pathway inhibitor\textsuperscript{16}. The transcription factor FoxQ1 has been shown to be required for surface mucous cell formation, with a loss of FoxQ1 leading to reduced surface mucous cell numbers\textsuperscript{17}. Surface mucous cells turn over every 2-3 days\textsuperscript{14}.

Parietal cells are the acid-secreting cell type of the stomach and can be located in any region of the corpus gland. Parietal cells contain a highly dense canalicular network from which acid is secreted into the lumen of the stomach\textsuperscript{18}. Parietal cells express H/K ATPase, parathyroid hormone-like hormone (PTHLH), and ADP-ribosylation factor 1 (ARF1)\textsuperscript{15}. The Hedgehog pathway and epidermal growth factor (EGF) are thought to play a key role in parietal cell differentiation, as cultures of canine parietal cells stimulated with either EGF or sonic hedgehog increased expression of H/K ATPase\textsuperscript{19}. The bone morphogenic pathway (BMP) also plays a role in parietal cell differentiation; a mouse model expressing the BMP antagonist noggin in parietal cells exhibited loss of parietal cells and increased proliferation\textsuperscript{20}. Parietal cells are generated from preparietal cells and turnover every 54 days\textsuperscript{14}.
Figure 1-2. Schematic of gastric epithelial differentiation. Schematic depicting the key pathways or transcription factors for development of differentiated cell types of the corpus and antral epithelium. Key transcription factors described in text.
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\textsuperscript{21}. \textit{Mist1} is a key transcription factor regulating this maturation. A genetic loss of Mist1 leads to structural abnormalities in chief cells and an increase in pre-neck transition cells\textsuperscript{21}. Chief cells are the longest-lived differentiated cell type of the stomach, with a half life of 194 days\textsuperscript{22}. Mucous neck cells express trefoil factor family 2 (TFF2) while chief cells express pepsinogen C (PGC) and gastric intrinsic factor (GIF)\textsuperscript{23–25}.

Scattered throughout the corpus gland are enteroendocrine cells, which are broadly marked by chromogranin A (CHGA)\textsuperscript{26}. The histamine-secreting enterochromaffin-like (ECL) cells are the primary enteroendocrine cell type in the corpus, but there are also ghrelin-, somatostatin-, and serotonin-secreting cells\textsuperscript{27}. There has not been a significant amount of work characterizing these cell types, but approximately 50% of CHGA-expressing enteroendocrine cells in the corpus, including all ECL and serotonin-expressing cells, originate from \textit{NeuroD}-expressing cells\textsuperscript{28}.

\textbf{Antral gland structure and cell types of the mouse stomach}

In the antral region, key features of gland architecture and cellular make-up differentiate this region from the corpus. Shorter gastric units resemble intestinal crypts, with stem cells housed in the base of the gland (Fig 1-3)\textsuperscript{29}. These antral stem cells (marked by leucine-rich repeat-containing G-protein coupled receptor 5 [LGR5]) differentiate into surface mucous, deep mucous and enteroendocrine cells (Fig 1-2). The antral epithelium undergoes turnover much faster than the corpus, with complete
Figure 1-3. Mouse antral gland. (A) Histological section of mouse antral gland stained with haematoxilin and eosin. (B) Cartoon depiction of mouse antral gland with various cell types identified with specific markers used in this study to identify the differentiated cell types. Mucin 5AC (MUC5AC), chloride channel accessory 1 (CLCA1), trefoil factor 2 (TFF2), griffonia simplicifolia II (GSII), chromogranin A (CHGA), gastrin (GAST), leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5). Scale = 25 µm.
turnover in 7-10 days\textsuperscript{30}. In the antrum the majority of enteroendocrine cells are descendants of \textit{Neurog3} expressing progenitors\textsuperscript{31}. The transcription factor SPDEF is required for mucous neck cell differentiation in the antrum, suggested from studies showing genetic mouse models lacking \textit{Spdef} had impaired terminal maturation of deep mucous cells and reduced numbers of secretory granules\textsuperscript{32}. Similar to the corpus, FOXQ1 is a necessary transcription factor for proper differentiation of deep mucous cells\textsuperscript{17}.

\textit{Features of the human stomach}

The human stomach exhibits some differences compared to the mouse stomach\textsuperscript{33}. The human stomach lacks a forestomach, but does include a glandular cardia region around the gastroesophageal junction. Human gastric glands are typically branched with multiple glands leading to a single luminal pit. While the mouse stomach is characterized by the restriction of parietal cells to the corpus, parietal cells in the human stomach are seen throughout the corpus and in over 50\% of antral glands\textsuperscript{33}. Human antral glands are subgrouped into three types: oxyntic-type (containing parietal and chief cells), antral type (containing G-cells and TFF2-positive mucous cells) and mixed-type (containing both parietal and G-cells). The human corpus is distinguished by the expression of ghrelin, and the antrum by gastrin\textsuperscript{33}.

\textit{Gastric stem cells}

The homeostasis of adult tissues relies on small populations of resident stem cells. These specialized cells are able to maintain themselves over long periods of time
(self-renewal) and generate all differentiated cell types of the resident tissue (multipotency). The balance between stem cell self-renewal and differentiation must remain under tight control to maintain proper development and avoid uncontrolled proliferation that may lead to cancer. Stem cells exist in two states: quiescent and active\textsuperscript{34}. Active stem cells undergo frequent division and contribute to normal epithelial homeostasis. Quiescent stem cells divide infrequently and typically contribute to epithelial cell renewal under injury conditions where active stem cells may be damaged. Both active and quiescent stem cells have been defined for the gastric epithelium\textsuperscript{29,35–37}.

Stem cells reside in specific microenvironments (niches) that support crucial stem cell maintenance. The localization of gastric stem cells was first identified in the 1940s, when \textsuperscript{32}P nucleotides were incorporated into nuclei of dividing cells, which appeared in the isthmus region of corpus glands\textsuperscript{38}. The investigators concluded that this region was the site of cellular renewal in undamaged tissue. These cells were characterized as “nondifferentiated cells”, with a high nucleus-to-cytoplasm ratio, open chromatin, lack of granules, and many free ribosomes with few mitochondria\textsuperscript{39}. Similar cells have been localized in the isthmus region of the antrum\textsuperscript{29}.

The concept of gland monoclonality, or the derivation of all mature lineages in a gland from a single stem cell, has been shown in the intestine to occur through neutral competition of stem cell progeny for niche space, or neutral drift\textsuperscript{40}. In the adult antrum, each gland houses 3-4 active stem cells in its base\textsuperscript{29}. Gastric glands also exhibit monoclonality, with an entire gastric gland being populated by a single stem cell. This conclusion comes from studies by Nomura et al.\textsuperscript{41} where they followed expression of an X-linked LacZ transgene, which is inactivated randomly in females, to observe the blue
(LacZ-positive) white patterning to determine if the gland was being fed by one or multiple stem cell. In adult mice, most units were completely blue or white, suggesting monoclonality\textsuperscript{41}. This finding was verified and expanded by the Barker lab with observation of LGR5-positive stem cells in the antrum drifting towards monoclonality over time\textsuperscript{42}.

A key approach to determining if a specific population of cells are stem cells is lineage tracing. This technique marks a single cell with a permanent genetic reporter; thus all the cell’s progeny will retain the mark resulting in labeled clones. Lineage tracing provides information about the number of progeny from a specific cell, their location, and the differentiated cell types that come from a specific cell\textsuperscript{43}. For gastric stem cells, a long-lived lineage trace is observed in an entire gastric gland and encompasses all differentiated cell types of either the antrum or corpus. The Clevers lab identified \textit{Lgr5} as a marker of antral stem cells by observing full gland lineage tracing with a \textit{Lgr5} gene-induced genetic mark that persisted for over a year\textsuperscript{29}.

\textit{Gastrointestinal organoids}

Recently, there have been significant advances in the \textit{in vitro} culture of mouse and human gastrointestinal epithelial cells. The intestinal organoid culture technique was pioneered by the Clevers’ lab and involves the establishment of intestinal stem cells in culture to form physiologically relevant 3D spheroids that thrive without the support of mesenchymal cells, and has been applied to other tissues such as the stomach (Fig 1-4)\textsuperscript{44}. Intestinal organoids exhibit crypts with morphology similar to the adult small intestine though they lack villi. Cellular proliferation,
For human and mouse gastric organoid establishment, glands are isolated from gastric tissue and plated in Matrigel. With the addition of specific growth factors, such as Wnt, R-spondin, and Noggin, spheroids contain differentiated cell types typical for the mouse stomach.
differentiation, and apoptosis in these organoids mimics in vivo cellular homeostasis\textsuperscript{44}. This system is a powerful tool due to the ability to sustain growth of intestinal stem cells in vitro for many months to years\textsuperscript{44}.

The development of organoids from single isolated cells has proven to be another key test to validate gastrointestinal stem cell identity. Single LGR5-expressing intestinal cells were demonstrated to form organoids in vitro, which present crypt-villus compartments and all differentiated cell types of the adult epithelium\textsuperscript{44}. Lgr5-expressing antral cells were also shown to form long-lived antral organoid cultures\textsuperscript{29}. The use of organoids is a powerful approach to test multipotency and stemness as well as epithelial-specific regulation of stem cell function.

Since the introduction of intestinal organoids by Sato et al\textsuperscript{44}, the technique has been adapted to other organs from the neonatal and adult mouse such as the liver\textsuperscript{45}, pancreas\textsuperscript{46}, and stomach\textsuperscript{29}, but also to human tissues from the stomach, small intestine, and colon\textsuperscript{47,48}. The organoid culture system has become an exciting new tool in the study of human development, disease, and homeostasis across numerous tissue types.

\textit{Markers of gastric stem cells}

A significant limitation of our current understanding of gastric stem cell regulation is the few robust markers identified for both active and quiescent gastric stem cells. These markers are useful for visualizing stem cells and for generation of cell-specific genetic tools to study how a marked cell population might contributes to gastric epithelial homeostasis. Below are summaries of defined gastric stem or progenitor cell markers. Importantly most of these conclusions were defined in a single mouse study.
and are generally not thoroughly characterized. A summary of gastric stem cell markers is provided in Table 1-1.

Similar to the intestine, the Wnt target gene *Lgr5* marks adult stem cells located at the base of the antral glands. There are 3-4 long-lived LGR5-positive stem cells located at the base of each gland that give rise to all differentiated cell types of the antral epithelium. *Lgr5*-expressing cells were found to be able to give rise to all cell types of the antrum with long-term renewal, with lineage tracing persisting over 620 days. Isolated LGR5-positive cells are also capable of generating and maintaining gastric organoids *in vitro* that give rise to antral cell types. LGR5-expressing cells are present throughout the gastric antrum, with few LGR5-expressing cells present in the corpus region, mainly confined to chief cells along the lesser curvature of the stomach. LGR5 also marks stem cell precursors in the corpus and antrum of immature mouse stomachs.

SOX2 marks uncommitted cells in the gastric corpus and antrum. Long-term lineage tracing from a *Sox2-CreERT2* transgenic mouse showed that *Sox2*-positive cells give rise to all differentiated cell types in both the corpus and antrum. SOX2 does not mark LGR5-positive cells. Long-term lineage tracing showed persistent tracing of both corpus and antral glands for over a year. Co-staining of SOX2 with the proliferation marker Ki67 showed that roughly half of the SOX2-positive cells are actively cycling. Analysis of *Sox2-CreERT2;ROSA26-Isl-eYFP* mice one day or one week after tamoxifen administration showed that some *Sox2*-positive cells are cycling and give rise to eYFP patches after one week, while other *Sox2*-positive cells appear to be slow cycling and remain as singly labeled cells.
Table 1-1. Description of genetic markers of mouse gastric stem cells.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mouse Model</th>
<th>Corpus or Antrum</th>
<th>Active or Quiescent</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lgr5</td>
<td>Lgr5-EGFP-ires-CreERT2</td>
<td>Antrum</td>
<td>Active</td>
<td>29</td>
</tr>
<tr>
<td>Sox2</td>
<td>Sox2-CreERT2</td>
<td>Both</td>
<td>Active</td>
<td>50</td>
</tr>
<tr>
<td>Cckbr</td>
<td>CCK2R-CreERT-BAC</td>
<td>Antrum</td>
<td>Active</td>
<td>51</td>
</tr>
<tr>
<td>Lrig1</td>
<td>Lrig-CreERT2</td>
<td>Both</td>
<td>Quiescent</td>
<td>36</td>
</tr>
<tr>
<td>Vil1</td>
<td>Vil-LacZ</td>
<td>Antrum</td>
<td>Quiescent</td>
<td>37</td>
</tr>
<tr>
<td>Tnfrsf19</td>
<td>Troy-eGFP-ires-CreERT2</td>
<td>Corpus</td>
<td>Quiescent</td>
<td>35</td>
</tr>
</tbody>
</table>
The gastrin receptor cholecystokinin B receptor (Cckbr also designated as Cck2r) labels long-lived actively cycling stem cells of the antrum. Analysis of Cck2r-CreERT-BAC transgenic mice crossed to a ROSA reporter mouse identified CCK2R-expressing cells near the base of antral glands above LGR5-expressing cells. Tamoxifen activation of gave rise to long-lived lineage tracing of antral glands, including all mature antral cell lineages.

LRIG1 has been suggested to be a quiescent intestinal stem cell marker based on slower proliferation rates than active LGR5-marked stem cells. Limited data has been published in regard to expression in gastric stem cells, but lineage-tracing studies showed long-lived tracing from a Lrig-CreERT2 transgene in both the corpus and antral regions of the mouse stomach.

A rare subpopulation of gastric progenitors was identified by a marked allele of the villin (Vil1) gene, Vil-LacZ. These LacZ-positive cells were localized in the antrum at or below the isthmus region of the gland. This is a quiescent population that does not normally proliferate or contribute to normal gastric epithelial lineages. However, after stimulation with the proinflammatory cytokine interferon-γ this population exhibits multilineage potential with lineage-traced cells populating entire antral glands. These cells are not seen in the corpus.

Tnfrsf19 (Troy) is a marker of a subset of fully differentiated chief and parietal cells in the gastric corpus. A Troy-eGFP-ires-CreERT2 knock-in mouse was generated and expression of eGFP was seen at the base of corpus glands in occasional chief and parietal cells. Crossing to a ROSA reporter strain showed rare lineage tracing of full corpus glands after four weeks that persisted for at least 1.5 years. Single Troy-positive
cells were shown to form gastric organoids \textit{in vitro}. In a damage model where proliferating cells were killed by 5-fluorouracil (5-FU), \textit{Troy}-positive cells showed accelerated expansion and the number of lineage tracing events increased 6-fold\textsuperscript{35}. 

\textit{Tff2-CreERT2} BAC transgenic was found to mark progenitors for a subset of gastric cell types in the corpus\textsuperscript{53}. \textit{Tff2-CreERT2} did not however mark a stem cell as lineage tracing was not observed in all cell types and the marks were not long-lived. Tff2-CreERT2 expressing cells in the corpus marked progenitors of chief and parietal cells, but not surface mucous or enteroendocrine cells\textsuperscript{53}.

\textit{Mist1} is a transcription factor expressed in differentiated chief cells\textsuperscript{21}. In a \textit{Mist1-CreERT2} mouse model challenged with a spasmolytic polypeptide-expressing metaplasia (SPEM)-inducing drug, lineage tracing showed that the emerging TFF2-positive SPEM lineage traced from the \textit{Mist1-CreERT2} cells. \textit{Mist1}-positive cells have the capacity to act as progenitors to reacquire proliferative ability and give rise to SPEM cells\textsuperscript{54}, but not all cell types of the epithelium, indicating that \textit{Mist1} is not a stem cell marker.

\textit{Pathways regulating gastric epithelial proliferation}

Various signaling pathways have been shown to be key regulators of epithelial proliferation and/or differentiation in the stomach. Below are short summaries of some of the most well characterized signaling pathways and their role in the stomach.

Hedgehog signaling was identified as a contributor to gastric physiology after observation that Hedgehog ligand (sonic hedgehog (Shh)) null mouse mutants expressed metaplastic changes in the stomach\textsuperscript{55}. The specific role Shh plays in gastric
maturation was difficult to identify due to Shh-null mouse death within 24 hours of birth. In the normal adult mouse stomach, Shh expression is high in the forestomach and corpus and lower in the antrum, with the greatest expression in parietal cells. In adult tissue, analysis is limited due to the lack of stomach-specific promoters for Cre recombinase. To test Shh function in specific cell types, Xiao et al. used the H/K-ATPase driven constitutive Cre to delete Shh in parietal cells. These mice develop hypergastrinemia and an expanded surface mucous cell region. These studies suggest that Hedgehog signaling is important for maintenance of proper gastric physiology, but a specific regulatory role for gastric stem cells remains unknown.

There is little known about the role of Wnt signaling in normal gastric homeostasis. Wnt is a key factor in maintenance of in vitro gastric organoid cultures and Lgr5, a marker of antral stem cells, is a Wnt target gene, suggesting that Wnt signaling is important for stem cell maintenance. Deletion of the Wnt negative regulators APC or GSK3 in the stomach led to rapid loss of parietal cells and fundic gland polyp formation. Deletion of APC from antral stem cells using Lgr5-EGFP-CreERT2 resulted in gastric adenoma formation. These studies suggested that Wnt signaling is key to maintaining normal gastric homeostasis and further analysis will be necessary to detail Wnt function in the stomach.

Various receptors and ligands of BMP signaling are expressed in both epithelial and mesenchymal cells of the stomach, including BMP2 in epithelial cells and BMP4 in non-epithelial myofibroblast-like cells. Loss of BMP signaling in early development impacts overall stomach patterning, with an expansion of the foregut region. Expression of the BMP antagonist noggin in parietal cells in the adult mouse resulted in
a loss of parietal cells, the development of transitional cells that express both mucus neck and zymogenic lineages, and activation of proliferation\textsuperscript{20}. These profound abnormalities in maturation and differentiation indicate that BMP signaling plays a crucial role in the regulation of normal gastric homeostasis.

TGF$\alpha$ is produced by the gastric mucosa and inhibits acid secretion, stimulates cell migration and proliferation after injury, and regulates gastric mucus levels\textsuperscript{62}. TGF$\alpha$ has been implicated in the pathogenesis of Menetrier's disease, a disorder of the stomach characterized by corpus gland hyperplasia\textsuperscript{63}.

Gastrin is a key hormonal inducer of acid secretion in the stomach. Gastrin also serves as a growth factor by stimulating epithelial proliferation and a thickening of the gastric mucosa\textsuperscript{64}. It has been suggested that the growth-promoting effect of gastrin in the corpus may be due to direct interaction of gastrin with progenitor cells, but this remains debatable\textsuperscript{51,65}.

1.2: THE NOTCH SIGNALING PATHWAY

Notch signaling is an evolutionarily conserved pathway that plays a critical role in multiple developmental programs at various stages. Notch signaling links the fate of neighboring cells, with physical interaction of membrane-bound Notch receptors with a ligand expressed in an adjoining cell (Fig 1-5). The outcome of Notch signaling is dependent on the cellular context, and can include proliferation, terminal differentiation, and lineage commitment. Examples of the variety of outcomes offered in various mammalian tissues have been summarized in Table 1-2. The Notch signaling pathway
Figure 1-5. Schematic of the Notch signaling pathway. Upon binding of a Notch ligand with a Notch receptor, the negative regulatory region of the Notch receptor undergoes a conformational change and exposes a cleavage site. ADAM10 cleaves the Notch extracellular domain from the Notch intracellular domain (NICD). A γ-secretase complex cleaves the NICD from the transmembrane domain to release it for translocation to the nucleus. Binding of NICD to the DNA-binding protein RBPJ releases co-repressors (CoR) and allows for association with co-activators (CoA, mastermind-like (MAML), p300) to lead to Notch target gene transcription.
Table 1-2. Role of the Notch signaling pathway in various organs/tissues.

<table>
<thead>
<tr>
<th>Organ/Tissue</th>
<th>Processes Regulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>Balance of gliogenesis and neurogenesis, stem cell maintenance, apicobasal polarity</td>
</tr>
<tr>
<td>Breast</td>
<td>Alveolar development, maintenance of luminal cell fate, regulation of basal cell proliferation</td>
</tr>
<tr>
<td>Craniofacial structures</td>
<td>Palate morphogenesis, tooth development</td>
</tr>
<tr>
<td>Ear</td>
<td>Defines sensory epithelium, hair and supporting cell fate decision</td>
</tr>
<tr>
<td>Esophagus</td>
<td>Regulates epithelial homeostasis</td>
</tr>
<tr>
<td>Eye</td>
<td>Fiber cell differentiation in lens development</td>
</tr>
<tr>
<td>Heart</td>
<td>Cardiac patterning, cardiomyocyte differentiation, valve development, ventricular trabeculation, outflow tract development</td>
</tr>
<tr>
<td>Hematopoietic system</td>
<td>Balances B-cell versus T-cell development, maintenance of stem cells, maintenance of myeloid homeostasis</td>
</tr>
<tr>
<td>Intestine</td>
<td>Controls proliferation and differentiation, stem cell maintenance</td>
</tr>
<tr>
<td>Kidney</td>
<td>Cell fate of podocytes and proximal tubules</td>
</tr>
<tr>
<td>Limbs</td>
<td>Apical ectodermal ridge formation and digit morphogenesis</td>
</tr>
<tr>
<td>Liver</td>
<td>Ductal plate formation, intrahepatic bile duct morphogenesis</td>
</tr>
<tr>
<td>Lungs</td>
<td>Lateral inhibition between tracheal cells</td>
</tr>
<tr>
<td>Muscle</td>
<td>Promotes transition of satellite cells to myogenic precursor cells and myoblasts</td>
</tr>
<tr>
<td>Neural crest</td>
<td>Controls patterning of neural crest precursors, regulates transition of Schwann cell precursor to Schwann cell, controls Schwann cell proliferation and inhibits myelination, melanocyte stem cell maintenance</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Directs endocrine cell differentiation, maintain endocrine precursor cells, inhibits terminal acinar cell differentiation, controls epithelial branching and bud size</td>
</tr>
<tr>
<td>Pituitary</td>
<td>Regulates pituitary growth/proliferation, melanotrope specification and gonadotrope differentiation</td>
</tr>
<tr>
<td>Placenta</td>
<td>Controls fetal angiogenesis, maternal circulatory system development, spongiotrophoblast development</td>
</tr>
<tr>
<td>Prostate</td>
<td>Epithelial differentiation and growth</td>
</tr>
</tbody>
</table>
| Sex organs and germ        | Maintenance of Leydig progenitor cells, regulation of}
<table>
<thead>
<tr>
<th>Organ</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>Regulates cell adhesion, controls proliferation, controls hair follicle or feather papillae differentiation and homeostasis&lt;sup&gt;109,110&lt;/sup&gt;</td>
</tr>
<tr>
<td>Spine</td>
<td>Somite segmentation&lt;sup&gt;111,112&lt;/sup&gt;</td>
</tr>
<tr>
<td>Spleen</td>
<td>Regulates generation of T lineage-restricted progenitors and marginal zone B-cell development, controls homeostasis of dendritic cells&lt;sup&gt;113&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stomach</td>
<td>Regulation of epithelial proliferation in corpus and antrum, determine cell fate choice in antrum, regulates stem cells in the antrum, regulates cell fission&lt;sup&gt;10,11,114&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thymus</td>
<td>Thymic morphogenesis, differentiation of gamma delta lineage T-cells&lt;sup&gt;69&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thyroid</td>
<td>Regulates the number of thyrocyte and C-cell progenitors and regulates differentiation and endocrine function of thyrocytes and C-cells&lt;sup&gt;115&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vasculature</td>
<td>Regulates arteriovenous specification and differentiation in endothelial cells and smooth muscle cells, regulates blood vessel sprouting and branching&lt;sup&gt;116&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
is important in the regulation of adult stem cells in the gut, skin, muscle, hematopoietic system, and lungs\textsuperscript{117}. The Notch pathway must be able to produce spatially and temporally appropriate responses, as Notch malfunction has been linked to diseases with altered cellular proliferation, such as cancer.

\textit{Notch receptors and ligands}

In canonical Notch signaling, transmembrane Notch receptors interact extracellularly with transmembrane Notch ligands on an adjacent cell. This initiates proteolytic cleavage of the Notch receptor to release the Notch receptor intracellular domain (NICD). There are four Notch receptors (NOTCH1-4) that are multidomain proteins with structures that have been conserved from invertebrates to humans\textsuperscript{118}. There are five Notch ligands, three from the Delta-like family (DLL1, DLL3, DLL4) and two from the Jagged family of Serrate homologs (JAG1, JAG2)\textsuperscript{119}. This allows for a significant number of unique receptor-ligand combinations, but there is little evidence for distinct responses from particular combinations. Instead, tissue and cell specific expression of receptors and ligands appears to account for the varying responses to Notch signaling.

\textit{Structure of Notch receptors and ligands}

The Notch receptor is composed of an extracellular domain (NECD) and intracellular domain (NICD) tethered by a transmembrane domain that can be cleaved to release NICD (Fig 1-6). NECD consists of 29 to 36 EGF homology repeats, which vary for different Notch receptors. These EGF repeats mediate interaction with ligands and can be modified post translationally to alter Notch function\textsuperscript{118}. NECD is followed by
Figure 1-6. Schematic of the Notch receptor structure. The extracellular region of the Notch receptor (NECD) is composed of 29 to 36 epidermal growth factor (EGF) repeats and the negative regulatory region (NRR), which is composed of three cysteine-rich Lin-12-Notch repeats (LIN) and a heterodimerization domain (HD). The Notch receptor intracellular domain (NICD) is composed of the RBPJk associated module (RAM), two nuclear localization sequences (NLS) flanking seven anykrin repeats (ANK), the transcriptional activator domain (TAD) and a C-terminal proline, glutamic acid, serine, threonine-rich (PEST) domain. Inhibitory antibodies can block Notch signaling by targeting the NRR of specific receptors.
the negative regulatory region (NRR), which prevents access to the S2 cleavage site in the absence of Notch ligands. The NRR is composed of three Lin-12-Notch repeats (LIN) and a hydrophobic region for receptor heterodimerization (HD)\(^ {120,121}\). Following the transmembrane domain, NICD is composed of the RBPJk association module (RAM), two nuclear localization sequences (NLS) flanking a seven ankyrin repeats (ANK) domain, a transcriptional activation domain (TAD) and a C-terminal proline/glutamic acid/serine/threonine-rich (PEST) motif\(^ {121}\). RAM and ANK are essential to allow NICD to target RBPJ in the nucleus, and PEST is a target for NICD degradation\(^ {121,122}\). The Notch ligands are transmembrane proteins that have an extracellular domain with EGF-like repeats and a N-terminal Delta/Serrate/lag-2 (DSL) domain, which is required for interaction with Notch receptors\(^ {122}\).

**Notch receptor processing**

Release of NICD requires multiple cleavage events of the Notch receptors. The first cleavage event, S1, is a Furin-dependent cleavage that occurs in NECD during trafficking through the Golgi complex\(^ {123}\). The NRR plays a crucial role in preventing subsequent S2 cleavage from occurring in the absence of ligand binding by conformational blocking of the cleavage site. Many studies have shown NRR to be the “activation switch” of the receptor through the discovery that receptors that lack the EGF-like repeats are functionally inert\(^ {124-126}\) and deletion or point mutation of the LIN module lead to gain-of-function phenotypes\(^ {127}\). Binding of a Notch ligand to a Notch receptor induces a conformational change that relaxes and unfolds the NRR, exposing the S2 cleavage site\(^ {128}\). S2 cleavage event is performed by an ADAM metalloprotease,
with multiple ADAM family members being implicated in Notch receptor cleavage events\textsuperscript{129–133}. However, more recent \textit{in vivo} data has definitively shown that ADAM10 is responsible for S2 cleavage\textsuperscript{133}. ADAM10 cleavage generates the activated intermediate termed the Notch Extracellular Truncation (NEXT)\textsuperscript{129,134}. NECD is trans-endocytosed into the signal-sending cell and can be recycled back to the plasma membrane or sorted into late endosomes\textsuperscript{135}. The tethered NEXT is then the site for S3 cleavage, which is completed by the γ-secretase complex that contains Presenilin\textsuperscript{136}. After S3 cleavage the NICD is released from the membrane and is free to translocate to the nucleus.

\textit{Notch intracellular domain}

Upon ligand binding and proteolytic processing of the Notch receptor, NICD is able to translocate directly to the nucleus. In the nucleus NICD forms a transcriptional complex with the DNA binding protein RBPJk through the RAM domain, Mastermind (MAML), and various transcriptional co-activators to activate expression of Notch target genes\textsuperscript{120,121}. In the absence of NICD, RBPJk forms a complex with co-repressors and histone deacetylases to prevent activation of Notch target genes\textsuperscript{120,121}. NICD in the nucleus is stabilized through phosphorylation by kinases such as glycogen synthase kinase 3β (GSK3β) and cyclin-dependent kinase 8 (CDK8)\textsuperscript{137–139}. NICD is targeted for proteasomal degradation by the E3 ubiquitin ligase SEL10\textsuperscript{140,141}. The Notch signaling pathway lacks amplification steps, with each NICD being consumed during signaling.

\textit{Notch target genes}
Notch signaling in different cell types reveals considerable variety in the immediate downstream Notch response. The best characterized Notch target genes belong to the hairy and enhancer of split-related (HESR) family that code for basic helix-loop-helix (bHLH) transcription factors. HESR genes play an important role in promoting cell fates by repressing genes such as atonal homolog 1 (Atoh1), acheate-scute like 1 (Ascl1) and Neurogenin families of bHLH transcriptional activators\textsuperscript{142,143}. C-myc, CyclinD, CDK5, and CDC25 have been shown to be Notch target genes in cells where Notch promotes proliferation\textsuperscript{144–147}.

**Notch in adult stem cell homeostasis**

Notch signaling has been well characterized as a key mediator of stem cell homeostasis across numerous tissues. Below are short summaries of the role of Notch signaling in maintenance of skin, muscle, hematopoietic, and lung stem cells.

The skin epidermis is a rapidly renewing epithelium that serves as a barrier from environmental factors and to prevent dehydration. The skin epidermis is comprised of four layers: basal, spinous, granular, and cornified, with basal cells capable of self-renewal and production of new cells to differentiate and move outwards\textsuperscript{148}. RBPJ is expressed throughout all four layers, but the Notch receptors NOTCH1, NOTCH2, and NOTCH3 are only present in the spinous layer\textsuperscript{149}. A mouse genetic model of Notch inhibition through *Rbpj* deletion resulted in an absence of spinous cells due to a lack of cell exit from the niche and differentiation from the basal layer\textsuperscript{150}. Interestingly, the loss of Notch signaling triggered hyperproliferation via cytokine production and led to a tumor-promoting environment, leading some to believe that Notch has a “tumor
suppressor” phenotype in the skin\textsuperscript{151}. The Notch signaling pathway does not play a role in maintenance of quiescent stem cells in the skin\textsuperscript{118}.

Adult muscle growth and repair is dependent on the specialized subset of muscle cells, the satellite cells\textsuperscript{152}. Satellite cells are thought to be a mix of stem cells and more committed progenitor cells. Normally in a quiescent state, satellite cells can be activated upon damage to the muscle fibers to fuse and make myofibers that regenerate the tissue\textsuperscript{148}. Various Notch components are expressed in satellite cells, such as DLL1, JAG1, NOTCH1, NOTCH2, NOTCH3 and Notch target genes such as \textit{Hes1} and \textit{Hey1}\textsuperscript{153,154}. Constitutive expression of Notch1 or the overexpression of DLL1 in cultured myogenic cells inhibited myogenic differentiation\textsuperscript{155–157}. Conversely, inhibition of the Notch signaling pathway through overexpression of the negative regulator Numb or administration of \(\gamma\)-secretase inhibitors promotes myoblast differentiation and stimulates formation of larger myotubes\textsuperscript{155,156,158}. These data indicate that Notch plays a key role in promoting proliferation of satellite cells while inhibiting terminal differentiation.

Hematopoietic stem cells in bone marrow give rise to myeloid and lymphoid precursors that differentiate to form red and white blood cells (T cells, B cells), megakaryocytes, and other cell types\textsuperscript{148}. Notch signaling is crucial for the generation of adult hematopoietic stem cells, with NOTCH1 and JAG1 required for development of differentiated cells\textsuperscript{159,160}. The role for adult stem cell homeostasis is unclear. The overexpression of Notch components results in the increased renewal of hematopoietic progenitors\textsuperscript{161}. However, knock out of Notch components such as NOTCH1 and JAG1 have no effect on hematopoietic stem cell homeostasis\textsuperscript{162}. It is known that Notch is essential for the maturation of the T cell lineage. Loss of Notch signaling by genetic
deletion of NOTCH1 or RBPJ results in a loss of T cells and an increase in B cells, showing Notch promotion of a binary cell fate decision between these two cell types\textsuperscript{162,163}. Notch is important for lineage commitment and terminal differentiation, but its role in maintenance of hematopoietic stem cells remains unclear.

The mammalian respiratory system consists of branched tubular structures that enable functional gas exchange. The lung epithelium contains undifferentiated basal cells, the stem cells of the lung epithelium, that give rise to differentiated ciliated and secretory cells\textsuperscript{164}. There is very little turnover of the lung epithelium in steady-state; self-renewal only occurs after damage to the tissue\textsuperscript{164}. At steady-state, NOTCH1, DLL1, JAG1, and JAG2 are expressed in the basal cells and, upon damage, expression is increased along with expression of Notch target genes\textsuperscript{165}. The expression of NICD in the lungs promoted differentiation of the secretory Clara cells, but not neuroendocrine cells, indicating Notch promotion of the secretory cell fate\textsuperscript{165}. This indicates that Notch is involved in directing cell fate choices in certain differentiated cell types of the lung epithelium.

\textit{Notch in disease}

The Notch signaling pathway plays a key role in stem cell maintenance in numerous tissues throughout mammals. However, mutations in various Notch components do occur and can lead to a number of diseases in humans. Table 1-3 summarizes disorders associated with mutations of Notch pathway components.
Table 1-3. Known Notch mutations associated with human diseases

<table>
<thead>
<tr>
<th>Disease</th>
<th>Symptoms</th>
<th>Notch Genes Associated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alagile Syndrome</td>
<td>Developmental abnormalities in heart, liver, eye, and skeleton</td>
<td>JAG1, NOTCH2</td>
</tr>
<tr>
<td>Cerebral autosomal dominant arteriopathy with subcortical infarcts and</td>
<td>Autosomal vascular disorders linked to ischemic strokes, dementia, and</td>
<td>NOTCH3</td>
</tr>
<tr>
<td>leukoencephalophathy (CADASIL) Syndrome</td>
<td>premature death</td>
<td></td>
</tr>
<tr>
<td>T cell acute lymphoblastic leukemia (T-ALL)</td>
<td>Anemia and enlargement of lymph nodes in liver or spleen</td>
<td>NOTCH1</td>
</tr>
<tr>
<td>Spondylocostal dysostosis</td>
<td>Vertebral segmentation defects, rib anomalies</td>
<td>DLL4</td>
</tr>
<tr>
<td>Hajdu-Cheney Syndrome</td>
<td>Bone resorption leading to osteoporosis</td>
<td>NOTCH2</td>
</tr>
</tbody>
</table>
1.3: REGULATION OF GASTROINTESTINAL STEM CELLS BY NOTCH

Notch intestinal function

The small intestine is similar to the gastric antrum, with LGR5-positive stem cells at the base of intestinal crypts giving rise to all differentiated cell types of the intestinal epithelium. Notch signaling affects both stem and differentiated cell types. DLL1 and DLL4 are the key Notch ligands and NOTCH1 and NOTCH2 are the key Notch receptors for intestinal cell regulation. Notch promotes intestinal stem cell proliferation and directs progenitor cells to the absorptive cell fate by inhibiting secretory cell differentiation. Loss of Notch signaling in the intestine through pharmacological inhibition of γ-secretase, or genetic knock-out of RBPJκ or NOTCH1/NOTCH2 resulted in loss of epithelial proliferation and secretory cell hyperplasia. Conversely, genetic activation of the Notch signaling pathway by overexpression of NICD was reported to increase epithelial proliferation and inhibit secretory cell differentiation.

The secretory cell fate inhibition by Notch occurs through negative regulation of the transcription factor atonal homolog 1 (Atoh1). Genetic mouse models have shown that constitutive Atoh1 expression promotes secretory cell differentiation, and Atoh1 gene deletion leads to the loss of the secretory cell lineage (goblet, endocrine, and Paneth cells).

Notch inhibition by γ-secretase inhibitors causes a downregulation of the intestinal stem cell marker olfactomedin 4 (Olfm4) in the intestine and transcription studies identified Olfm4 as a direct Notch target gene. Olfm4 has been shown to be a specific intestinal stem cell marker. The observation that Notch signaling directly
targets *Olfm4* expression demonstrates that Notch signaling is in the intestinal stem cell.\(^3\)

**Notch gastric function**

Compared to intestinal stem cells, the role of Notch in maintenance of gastric stem and epithelial cell homeostasis has not been well characterized. Kim et al. first outlined the role of Notch signaling in both the developing and adult stomach with findings described below\(^{10}\). Utilizing *in situ* hybridization, they showed multiple Notch ligands and receptors were expressed throughout the mouse embryonic epithelium, including *Notch1, Jag2, and Dll3*, as well as the Notch target gene *Hes1*. After administration of a γ-secretase inhibitor to block Notch signaling in adult mice, they observed a marked reduction of epithelial proliferation in both the corpus and antrum, which was accompanied by a significant increase in both mucous and enteroendocrine cells in the antrum. To investigate the consequence of Notch over-activation, a genetic mouse model was used that constitutively expressed NICD in parietal cells. These Notch-activated parietal cells dedifferentiated and had stem-like characteristics, giving rise to glands containing all cell types of the corpus epithelium. Over time, the Notch-activated corpus formed hyperproliferative adenomas with focal activation of the Wnt pathway. However, in this study, when NICD was constitutively expressed in LGR5 cells in the antrum, proliferation was not affected and adenomas did not develop. From these findings Kim concluded that Notch signaling is present in both the developing and the adult stomach and is required for maintaining gastric epithelial homeostasis\(^{10}\).
Demitrack et al. established an important role for Notch signaling to regulate LGR5 antral stem cells\textsuperscript{11}. Using pharmacological Notch inhibition through a $\gamma$-secretase inhibitor, proliferation of LGR5-positive stem cells was decreased. Using the same genetic model of Notch activation as the Kim study to drive NICD expression in Lgr5-positive cells, they observed increased antral epithelial and stem cell proliferation, contrary to the previous study. The NICD constitutive Notch activation model exhibited an increase in gland fission events and a rapid generation of monoclonal glands, indicating an increase in the number of stem cells per gland. Importantly, chronic Notch activation induced undifferentiated, hyper-proliferative polyps, suggesting that Notch hyper-activation in gastric stem cells may contribute to gastric tumorigenesis\textsuperscript{11}.

Analysis of differentiation in the Demitrack study showed that Notch inhibition increased all differentiated cell types of the antrum, including surface and deep mucous cells and enteroendocrine cells. Conversely, genetic Notch activation led to a decrease in all differentiated cell types\textsuperscript{11}.

These studies have highlighted the importance of Notch signaling in maintaining gastric epithelial homeostasis and the potential for Notch signaling to contribute to gastric cancer development. However, key questions remain in how Notch achieves this regulation. 1) What is the mechanism through which Notch regulates gastric epithelial homeostasis and 2) which pathway components are involved? These are the questions investigated in this thesis.

\textit{Expression of Notch receptors and ligands in gut}
The roles of individual Notch receptors and ligands are starting to be characterized. The adult intestine expresses Notch1, Notch2, and Notch3 receptors and Dll1, Dll4, Jag1, and Jag2 ligands. Notch1, Jag1, and Jag2 were expressed in the lower third of all crypts, in the proliferative region, while Notch2 was expressed in rare cells in select crypts. Notch1 was also abundantly expressed in the lamina propria, and both Notch1 and Notch2 showed expression in the underlying muscle layers. Notch3 was expressed primarily in the vasculature. To determine which cells express the Notch receptors, a set of knock-in mice was developed in which the Cre-estrogen receptor binding domain fusion was knocked-in to the first exon of each of the four Notch receptors (Notch1-4) and crossed to a floxed ROSA reporter strain. Notch3 and Notch4 were not expressed in the intestinal epithelium, but Notch1 and Notch2 gave rise to long-lived labeled intestinal clones suggesting that these two receptors are expressed in intestinal stem cells. The Notch1-Cre construct induced far greater numbers of lineage stripes compared to the Notch2-Cre expressing stem cells.

Less is known about Notch receptor and ligand expression in the stomach. Northern blot analysis show gene expression of Notch1, Notch2, Notch3, Jag1, Jag2, and Dll1 in both the forestomach and corpus region of the rat stomach. Analysis of the fetal mouse stomach showed expression of Notch1, Jag2, and Dll3 by in situ hybridization in the epithelium at embryonic day 17. My studies aimed to fill this gap by investigating the localization and function of the Notch receptors in the stomach.

*Notch receptors and ligands in intestinal epithelial homeostasis*
There have been various reports as to the role of the Notch receptors in maintaining intestinal epithelial homeostasis. Early studies indicated that NOTCH1 and NOTCH2 function redundantly, and only simultaneous loss of both receptors resulted in reduced epithelial proliferation and conversion of crypt progenitors into post-mitotic goblet cells, mimicking what is seen through γ-secretase inhibition. The development of specific inhibitor antibodies, detailed below, against NOTCH1 or NOTCH2 expanded this finding to show that NOTCH1 plays a more dominant role in epithelial homeostasis. Inhibition of NOTCH1 alone was sufficient to induce goblet cell metaplasia; however, the effect was mild compared to inhibition of both NOTCH1 and NOTCH2. In contrast, inhibition of NOTCH2 alone did not induce any proliferative or goblet cell phenotype. This indicates that NOTCH1 and NOTCH2 function redundantly in intestinal cell differentiation, although inhibition of NOTCH1 is sufficient to reveal a partial phenotype. Similar results were observed with a genetic model of Notch1 and/or Notch2 deletion. In addition, impaired crypt regeneration after radiation was observed in both Notch1-deleted and Notch2-deleted intestines, suggesting a need for higher Notch activity post-injury. Also, gene expression profiling of isolated LGR5 intestinal stem cells demonstrated Notch1 expression. Combined, these findings suggest that NOTCH1 is the primary receptor regulating intestinal stem cell homeostasis, and that both NOTCH1 and NOTCH2 regulate epithelial cell proliferation and differentiation in the adult intestine.

Investigation of the Notch ligands in the small intestine used genetic mouse models to deleteDll1, Dll4, and/or Jag1. Deletion of Jag1 or Dll4 did not alter intestinal epithelial homeostasis, but deletion of Dll1 did result in increased numbers of goblet
cells without effect on progenitor proliferation\textsuperscript{5}. However, deletion of both \textit{Dll1} and \textit{Dll4} led to loss of epithelial proliferation and conversion of progenitors into goblet cells, mimicking global Notch inhibition phenotype. This indicates that DLL1 and DLL4 are required for the maintenance of intestinal progenitors and stem cells\textsuperscript{5}. Paneth cells express DLL4 and a subset of transit-amplifying cells have been shown to express DLL1\textsuperscript{176,177}. Thus, DLL1 and DLL4 are the key Notch ligands in the intestinal stem/progenitor cells for epithelial and stem cell homeostasis.

\textit{Inhibition of Notch receptors by specific antibodies}

The four Notch receptors are expressed in various mammalian tissues to direct cell fate decisions and growth. Although γ-secretase inhibitors have progressed as potential therapeutics for Notch-related pathologies, they fail to distinguish between individual Notch receptors and have targets other than the Notch-signaling pathway. In order to elucidate independent functions of discrete Notch receptors, Wu \textit{et al.} developed inhibitory antibodies directed against the NRR of either NOTCH1 or NOTCH2\textsuperscript{175}. Utilizing phage display to generate antibodies targeting the NRR sequence, they showed that the antibodies potently inhibit NOTCH1 or NOTCH2 of either the human or mouse orthologue. Both antibodies inhibited signaling induced through different Notch ligands, including JAG1, JAG2, DLL1, and DLL4, indicating that inhibition occurs irrespective of the ligand. Examination of T cells and splenic marginal zone B (MZB) cell development proved functionality of specific receptor inhibition, because these processes depend specifically on Notch1 or Notch2, respectively. Treating mice with anti-Notch1 antibody specifically decreased T cells, while anti-
Notch2 decreased MZB cells, demonstrating the paralogue-specific inhibition\textsuperscript{175}. One main goal of the development of these antibodies was to circumvent the intestinal toxicity associated with γ-secretase inhibitors. Accordingly, administration of the Notch1-inhibitory antibody produced a partial goblet cell hyperplasia without fatal intestinal toxicity seen with γ-secretase inhibitors, representing a breakthrough over existing pan-Notch inhibitors for cancer therapeutics\textsuperscript{175}. I utilized these antibodies to study receptor function in the gastric epithelium.

\textit{Mouse models of Notch reporters}

Multiple tools have been developed to better understand the role of Notch signaling and the components involved in various tissues. Below are descriptions of genetically engineered mice used in this study to better understand the role of Notch signaling in the mouse stomach. A summary of the mouse models and schematic of the genetic constructs are present in Table 1-4 and Fig 1-7.

The Hadjantonakais lab created a unique Notch signaling reporter mouse model that utilizes a CBF1 (also known as RBPJκ) DNA binding site to provide single-cell resolution visualization of cells undergoing Notch signaling\textsuperscript{178}. This construct placed CBF1 binding sites into a promoter driving a human histone H2B nuclear localization sequence linked to the yellow fluorescent protein Venus to generate a transgenic mouse strain (CBF:H2B-Venus). This strain faithfully recapitulates Notch signaling in embryonic and adult tissue and is the first strain in which individual cells transducing a Notch signal can be easily visualized\textsuperscript{178}. This strain was used in Chapters 2 and 3 to identify cells in the adult mouse stomach that were undergoing Notch signaling.
**Table 1-4. Mouse models to detect Notch signaling and receptor expression**

<table>
<thead>
<tr>
<th>Mouse Model</th>
<th>Reports</th>
<th>Constitutive or Inducible</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBF:H2B-Venus</td>
<td>Active Notch signaling</td>
<td>Constitutive</td>
<td>178</td>
</tr>
<tr>
<td>Notch1-CreERT2 and Notch2CreERT2</td>
<td>Notch1 or Notch2 receptor expression</td>
<td>Inducible</td>
<td>4</td>
</tr>
<tr>
<td>Nip1-CreERT</td>
<td>Active Notch1 signaling</td>
<td>Inducible</td>
<td>179</td>
</tr>
</tbody>
</table>

**Figure 1-7. Schematic of genetic constructs for mouse models of Notch signaling and receptor expression.** Mouse models described in text and Table 1-4.
As mentioned previously, the Artavanis-Tsakonas lab created a set of knock-in mice to identify cells that express the Notch receptors\(^4\). Transgenic mice were created by knocking-in the Cre-estrogen receptor binding domain fusion (Cre-ER) to the first exon of each of the four Notch receptors. When crossed to a transgenic reporter strain (such as *ROSA-LacZ* or *ROSA-Tomato*) and activating the Cre-ER by tamoxifen treatment, the expression pattern of each of the four Notch receptors can be observed. Longer chase periods after Cre-ER activation can allow for identification of stem cells expressing Notch receptors through lineage tracing. These transgenic lines can be used for genetic recombination in specific Notch receptor-expressing cells by crossing the mice to a transgenic line with a gene of interest flanked by lox-P sites. This set of knock-in mice identified *Notch1* and *Notch2* expression in stem cells of the intestine, as described above\(^4\), with *Notch1* having a more robust number of lineage tracing events compared to *Notch2*. I used these strains in Chapters 2 and 3 to map the expression of the Notch1 and Notch2 receptors in the adult mouse stomach.

The Kopan lab created a mouse model to detect active Notch signaling through the *Notch1* receptor\(^{179}\). The genetic mouse model, named *NIP1-CreERT*, was engineered to replace NICD in the *Notch1* receptor with an inducible Cre recombinase. This results in Cre activity being governed by the ligand-induced proteolysis of Notch1. By crossing to a reporter strain, such as *ROSA-Tomato* and tamoxifen activation of the Cre, both expression and activity of *Notch1* can be visualized\(^{179}\). This model was used by the Samuelson laboratory to identify *Notch1* expression and function in antral stem cells\(^{11}\).
Notch receptors and ligands in human gastric cancer

There is evidence that disregulation of the Notch signaling pathway can contribute to gastric cancer progression. Gastric cancer is the second leading cause of cancer deaths worldwide and 5-year survival rates are less than 40%\(^1\). There are two types of gastric cancer based on histology: intestinal and diffuse. Intestinal type is characterized by gland-like tubular structures formed by cohesive neoplastic cells. Diffuse type is characterized by a poorly differentiated tumor that infiltrates the gastric wall. The cells of origin have not been determined, but gastric stem cells are strong candidates due to their high-proliferative potential and, as a regulator of gastric stem cell homeostasis, the Notch pathway may play a role in cancer initiation\(^2\).

Numerous studies have identified a correlation between human gastric cancer samples and Notch signaling, which are summarized in Table 1-5. One study found that levels of Jag1 correlated with aggressiveness of human gastric cancer and that overexpression of NOTCH1 NICD promoted the colony-forming ability of a human stomach adenocarcinoma cell line\(^3\). Another study found that NOTCH1 was rarely expressed in normal human gastric mucosa and was highly upregulated in premalignant cancer tissues, and that NOTCH2 was detected in all four tested gastric cancer cell lines\(^4\). NOTCH1, NOTCH3, JAG1, and JAG2 expression was found to be increased significantly in tumor compared to normal tissue in another study, with NOTCH3 and JAG2 associated with intestinal type gastric cancer\(^5\). In a different study the expression of NOTCH1, NOTCH2, DLL4, and HES1 was significantly higher in tumor tissues compared to normal, with NOTCH1 and JAG1 expression correlated to poor prognosis\(^6\). Together these studies, although each limited in scope, suggest that
Table 1-5. Notch signaling components associated with gastric cancer

<table>
<thead>
<tr>
<th>Gene</th>
<th>Role in gastric cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLL4</td>
<td>Expression of DLL4 in human gastric cancer cell line promoted proliferation, migration, and invasion$^{186}$</td>
</tr>
</tbody>
</table>
| NOTCH1| Expression increased in gastric tumors compared to normal$^{184}$  
Expression of NOTCH1-NICD promoted colony forming ability and xenograft tumor growth of stomach gastric cancer stem cells$^{182,187}$  
NOTCH1 upregulated with intestinal-like phenotypes of gastric lesions$^{183}$ |
| NOTCH2| Correlated with intestinal and diffuse type gastric cancer$^{183}$  
Expression of NOTCH2-NICD promoted human gastric cancer cell proliferation and xenograft tumor growth$^{188}$ |
| NOTCH3| Expression increased in gastric tumors compared to normal, associated with intestinal/glandular differentiation of gastric carcinoma cells$^{184}$ |
| NOTCH4| Expression in gastric cancer cell line promoted growth$^{189}$ |
| JAG1  | Expression increased in gastric tumors compared to normal$^{184}$ |
| JAG2  | Expression increased in gastric tumors compared to normal, associated with intestinal/glandular differentiation of gastric carcinoma cells$^{184}$ |
| DLL1  | Expression associated with diffuse and mixed type of gastric cancer$^{190}$ |
Notch components are upregulated in gastric cancer. Definitive knowledge as to the role of specific Notch receptors and ligands in gastric tumorigenesis is still limited.

1.4: THESIS OVERVIEW

The Notch signaling pathway has been identified as a key regulator of gastric stem and progenitor cell homeostasis. Notch signaling promotes antral stem cell proliferation and directs cell fate choice, and constitutive Notch activation leads to gastric polyp formation\textsuperscript{10,11}. However, the receptors or ligands involved in Notch regulation of gastric stem cells have not been identified.

This thesis project aimed to describe the role of the Notch receptors in the regulation of gastric stem and epithelial cell dynamics. I have approached this using mouse models to map cells that are Notch signaling and determine where NOTCH1 and NOTCH2 are expressed. Functional analysis of the NOTCH1 and NOTCH2 receptors was investigated by pharmacologic inhibition of Notch receptors in mouse models. Mouse and human gastric organoids were used to uncover the role of the Notch receptors in epithelial cells.

In Chapter 2, I investigated the function of Notch receptors in antral epithelial differentiation and stem cell maintenance with inhibitory antibodies directed against NOTCH1 or NOTCH2. I found that combined inhibition of NOTCH1 and NOTCH2 mimics pan-Notch inhibition from γ-secretase inhibitor treatment. I showed reduced epithelial and stem cell proliferation and increased apoptosis. My studies showed that NOTCH1 and NOTCH2 are the primary Notch receptors regulating antral epithelial homeostasis. Inhibition of both NOTCH1 and NOTCH2 led to increased differentiation of
all gastric antral lineages. Notch inhibition also led to the unique finding that cells in the base and surface of the glands remodeled with Notch inhibition, increasing expression of secretory products from other regions of the gastrointestinal tract, including the corpus and intestine. Analysis of mouse and human organoids showed that Notch signaling through NOTCH1 and NOTCH2 is intrinsic to the epithelium and regulates growth of human and mouse gastric stem cells *in vitro*.

In Chapter 3, I investigated the role of Notch signaling in the maintenance of corpus epithelial cells using pharmacologic inhibition of the Notch signaling pathway and inhibitory antibodies against the NOTCH1 or NOTCH2 receptors. Similar to the antrum, inhibition of NOTCH1 and NOTCH2 mimics the reduction in proliferating epithelial cells seen with pan-Notch inhibition, indicating that these are the key receptors involved in corpus epithelial homeostasis. No changes to differentiated cell types were observed *in vivo*, but this may be a consequence of the longer turnover time of corpus epithelial cells coupled with the short timepoint of pharmacologic Notch inhibition required due to the lethality of global Notch inhibition. I observed a decrease in mouse and human corpus organoid growth with pan-Notch inhibition and NOTCH1 and/or NOTCH2 inhibition, indicating that Notch signaling is intrinsic to corpus epithelial cells.

In Chapter 4, I outline the protocol I established for mouse and human gastric organoid culture. I characterize growth conditions and a histological analysis of established organoids. I investigated the role of Notch signaling on mouse corpus and antral organoid growth and found that antral organoids are significantly more sensitive to Notch inhibition compared to corpus organoids.
In Chapter 5, I have put my work in the context of the field and demonstrate how these findings provide important insights into the regulation of gastric stem and epithelial cells. I propose future directions and experiments that will further characterize and understand the role of Notch signaling on maintenance of gastric stem cells and how those findings can be used in a therapeutic context.

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2.1: SUMMARY

Objective

We tested the ability of Notch pathway receptors Notch1 and Notch2 to regulate stem and epithelial cell homeostasis in mouse and human gastric antral tissue.

Design

Mice were treated with the pan-Notch inhibitor dibenzazepine (DBZ) or inhibitory antibodies targeting Notch1 and/or Notch2. Epithelial proliferation, apoptosis and cellular differentiation were measured by histological and molecular approaches. Organoids were established from mouse and human antral glands; growth and differentiation were measured after treatment with Notch inhibitors.

Results
Notch1 and Notch2 are the predominant Notch receptors expressed in mouse and human antral tissue and organoid cultures. Combined inhibition of Notch1 and Notch2 in adult mice led to decreased epithelial cell proliferation, including reduced proliferation of LGR5 stem cells, and increased apoptosis, similar to the response to global Notch inhibition with DBZ. Less pronounced effects were observed after inhibition of individual receptors. Notch pathway inhibition with DBZ or combined inhibition of Notch1 and Notch2 led to increased differentiation of all gastric antral lineages, with remodeling of cells to express secretory products normally associated with other regions of the gastrointestinal tract, including intestine. Analysis of mouse and human organoids showed that Notch signaling through Notch1 and Notch2 is intrinsic to the epithelium and required for organoid growth.

Conclusions

Notch signaling is required to maintain gastric antral stem cells. Notch1 and Notch2 are the primary Notch receptors regulating epithelial cell homeostasis in both mouse and human stomach.

2.2: INTRODUCTION

The adult gastric epithelium is constantly renewed due to a population of actively cycling stem cells located in the gastric glands. These stem cells generate daughter cells that, upon exiting the stem cell niche, differentiate into the various epithelial cell lineages of the stomach. In the distal, antral stomach, active stem cells express the R-spondin receptor LGR5, which also marks stem cells in the intestine and other tissues\(^1,2\). Antral LGR5 stem cells give rise to all antral lineages, including surface
mucous cells, endocrine cells and deep mucous cells. The signaling pathways regulating gastric stem cell proliferation and differentiation are currently poorly understood.

Notch signaling is well described to maintain intestinal stem cells\(^3\)\(^-\)\(^7\) and recent studies suggest that gastric stem cells are similarly regulated by Notch\(^8\)\(^,\)\(^9\). In the stomach, pan-Notch inhibition led to reduced gastric stem and epithelial cell proliferation and increased differentiation of mucous and endocrine cell lineages. In contrast, activation of Notch through constitutive expression of the Notch Intracellular Domain (NICD) induce stem cell proliferation, gland fission and ultimately hyper-proliferative polyps\(^8\)\(^,\)\(^9\). Furthermore, increased expression of Notch signaling components has been associated with gastric cancer, suggesting Notch pathway involvement\(^10\)\(^,\)\(^11\).

Four Notch receptors (Notch1-4) exist in vertebrates that are single-pass transmembrane proteins\(^12\). Receptor signaling involves proteolytic receptor cleavage to release the intracellular signaling component NICD, which activates target gene transcription, such as those in the Hes and Hey families\(^13\). Notch1 and Notch2 are the primary receptors involved in intestinal stem cell homeostasis, with Notch1 having a predominant function\(^5\)\(^,\)\(^7\)\(^,\)\(^14\)\(^,\)\(^15\). Global pharmacological Notch inhibition leads to intestinal toxicity\(^3\), but inhibition of Notch1 alone revealed a partial Notch-inhibition phenotype while avoiding major toxicity\(^7\)\(^,\)\(^14\)\(^,\)\(^15\).

The specific Notch receptors regulating the stomach have not been described. In this study we examined the role of Notch receptors in epithelial and LGR5 stem cell homeostasis in the gastric antrum of genetic mouse models. We find that Notch1 and Notch2 are key regulators of stem cell proliferation, differentiation and apoptosis.
Furthermore our studies demonstrate that both Notch1 and Notch2 function to regulate growth of antral organoid cultures generated from human and mouse tissue.

2.3: METHODS

Mice

Mice of both sexes aged 2-3 months were used. Lgr5-EGFP-IRES-CreERT2 (Lgr5-GFP)² (Jackson Labs #008875), Notch1-CreERT2SAT (N1Cre) and Notch2-CreERT2SAT (N2Cre)¹⁶, CBF:H2B-Venus¹⁷ (Jackson Labs #020942) and ROSA26-CAG-LSL-tdTomato-WPRE (ROSA-Tom)¹⁸ (Jackson Labs #007909) mice were previously described. All mice were on a C57BL/6 background except for CBF:H2B-Venus, which was on a mixed background (CD1 and FVB/N). Mice were housed under specific pathogen-free conditions in automated watered and ventilated cages on a 12-hour light/dark cycle. Experimental protocols were approved by the University of Michigan Committee on the Use and Care of Animals. For lineage tracing Notch1- or Notch2-expressing cells, N1Cre; ROSA-Tom and N2Cre; ROSA-Tom mice were treated with either 1 injection of tamoxifen (1mg/20g body weight) followed by a 3-day chase or 5 daily injections of tamoxifen 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 Lgr5-GFP mice once per day for 5 days, with tissue collected the sixth day. Humanized
IgG1 neutralizing monoclonal antibodies specific for the Notch1- or Notch2-negative regulatory region (αN1 or αN2), or an irrelevant control IgG1 antibody interacting with herpes simplex virus gD protein (αGd) were described previously\textsuperscript{15}. 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 \textit{in vitro} treatment of mouse and human organoids, the gamma-secretase inhibitor N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-(S)-phenylglycine t-butyl ester (DAPT,1μM; EMD4Biosciences, Gibbstown, NJ, USA), αGd, αN1 or αN2 (10µg/mL) were added to culture media and renewed every other day for 5 days in established organoid lines.

\textit{Tissue Collection and Histological Analysis}

Mice were fasted overnight with free access to water before tissue collection. For some experiments, mice were injected with 5-ethyl-2'-deoxyuridine (EdU, 25 mg/kg, Invitrogen, Grand Island, NY, USA) 1.5 hr prior to tissue collection. Stomachs were processed for cryo and paraffin histology as described\textsuperscript{9}. For analysis of proliferation, EdU incorporation was visualized with the Click-iT EdU Alexa Fluor 488 Imaging Kit (Life Technologies, Carlsbad, CA, USA). Tissue sections were incubated with primary antibodies (Supplementary Table 2-1) followed by appropriate secondary antibodies (1:400, Invitrogen) and mounted with ProLong Gold containing 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen) as described previously\textsuperscript{18}. To analyze proliferating LGR5 stem cells, sections were immunostained for GFP (chicken) and Ki67 as described\textsuperscript{9}. Antral glands were isolated and immunostained as previously
described. Imaging by digital microscopy was done using a Leica SP5X Inverted 2-Photon FLIM Confocal Microscope or a Nikon E-800 Microscope.

For ultrastructural analysis, tissue was fixed and Epon embedded as described. Electron micrographs were captured using a JEOL JEM 1400 plus electron microscope.

**Gene Expression Analysis**

RNA was isolated from antral tissue or gastric organoids as previously described. RNA was isolated from human antral 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 RT-PCR was performed as described using primers listed in Supplemental Table 2-2, normalized to Gapdh (mouse) or ACTB (human).

**Gastric Organoid Culture**

Mouse gastric organoid culture was carried out as previously described. In brief, antral glands were resuspended in Matrigel (Corning, Tewksbury, MA, USA) and overlaid with culture media (50% L-WRN conditioned media, Advanced DMEM/F12, 10% FBS, 1X Pen-Strep, 2 mM L-Glutamine), with the addition of Y-27632 (10µM, Tocris, Bristol, United Kingdom) only upon initial plating. L-WRN conditioned media containing Wnt3a, R-spondin3, and Noggin was generated as described. Human antral tissue (surgical resection or Gift of Life) was obtained under Institutional Review Board approved protocols and organoids were established as described with modifications. 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 hr at 4°C on a rocking platform, vigorously shaken for 1 min to release glands and pelleted at 100 g for 5 min. Glands were resuspended in Matrigel and after 30 min at 37°C, culture media described above with the addition of Y-27632 and SB431542 (10 µM, Tocris Bioscience) was added to each well. Media was renewed every other day. Studies were performed in 3 independent established organoid lines that had been passaged at least 3 times before analysis.

*Morphometrics*

Morphometric analysis used ImageJ software (1.46r, Wayne Rasband, NIH, USA). For EdU incorporation, cleaved caspase-3 cells and gastrin cells, the entire length of the antrum for each animal was imaged (n=4-6 animals per group) and cell counts were normalized to epithelial length (µm). For LGR5 stem cell proliferation, the number of GFP/Ki67 double-positive cells per gastric antral gland was counted in αGd control (n=833 glands), αN1 (n=674 glands), αN2 (n=662 glands) and αN1+αN2 (n=548 glands) (n=5-7 mice/group). For organoid measurements, the area of at least 300 organoids per treatment was measured and data are presented as fold change compared to control (vehicle or αGd) organoids.

*Statistics*

GraphPad Prism software was used for statistical analysis. Quantitative data are presented as mean ± SEM and analyzed using Student’s t-test (comparing DBZ/DAPT
to vehicle) or 1-way ANOVA with Dunnett’s or Tukey’s post-hoc test (comparing $\alpha$N1, $\alpha$N2, $\alpha$N1+$\alpha$N2 and $\alpha$Gd). qRT-PCR data are expressed as mRNA fold-change vs. control (vehicle or $\alpha$Gd) with $P<0.05$ considered significant. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$. 
2.4: RESULTS

Notch signaling in antral stem cells

We used the CBF:H2B-Venus reporter mouse to identify cells undergoing Notch signaling in the gastric antrum. This transgenic mouse expresses a nuclear fluorescent protein in response to Notch receptor activation\textsuperscript{17}. Isolated antral glands showed scattered Venus-positive cells that were primarily located in the gland base (Fig 2-1A). Costaining showed Venus expression in differentiated cell types, including CHGA-expressing enteroendocrine cells (Fig 2-1B) and DCLK1-expressing tuft cells (Fig 2-1C). Crossing the Notch reporter strain to Lgr5-GFP mice showed that some of the nuclear Venus-positive cells were also marked with cytoplasmic GFP, indicating Notch signaling in antral LGR5 stem cells (Fig 2-1D).

Gene expression analysis for the four Notch receptors showed that Notch1 and Notch2 were the primary Notch receptors expressed in full thickness antral tissue and isolated glands (Fig 2-1E, Supplementary Fig 2-1). To test if Notch receptors are present in antral stem cells, we performed a lineage tracing experiment with mice expressing Notch receptor-CreERT2 genes crossed to ROSA-Tom mice. We examined N1Cre;ROSA-Tom and N2Cre;ROSA-Tom mice 3 days and 2 weeks post-tamoxifen treatment. Tomato-marked epithelial cells were evident in the antral gland base of N1Cre;ROSA-Tom and N2Cre;ROSA-Tom mice at 3-days post induction (Fig 2-1F, arrows). At 2 weeks post-tamoxifen epithelial lineage stripes were detected in the antrum of N1Cre;ROSA-Tom mice (arrows), suggesting that Notch1 is expressed in antral stem cells. The lack of lineage stripes in N2Cre;ROSA-Tom mice suggests that this receptor is expressed in short-lived cells.
Figure 2-1. Notch signaling in gastric antral cells. (A-D) Antral glands isolated from CBF:H2B-Venus mice (green, nuclear) co-stained for (B) chromogranin A (CHGA) (red), (C) doublecortin like kinase 1 (DCLK1) (red), or (D) Lgr5-GFP (green, cytoplasmic) and DAPI (blue). Insets show higher-powered views for each channel. (E) Notch receptor expression was determined by qRT-PCR analysis of total RNA isolated from full-thickness antral tissue or antral glands (mean ± SEM; n=3-5 mice). ND = Not detected. (F) Confocal imaging of paraffin sections from N1-Cre;Tom and N2-Cre;Tom mice co-stained for RFP (red), E-cadherin (green) and DAPI (blue) 3 days or 2 weeks after tamoxifen activation of Cre recombinase. Arrows and arrowheads indicate epithelial and non-epithelial Tomato expression, respectively. (G) Frozen tissue sections from CBF:H2B-Venus mice co-stained for the endothelial marker PECAM-1 (left, red) or the smooth muscle cell marker α-SMA (right, red). Co-stained cells in boxed regions are marked (arrowheads) in higher-powered insets. Scale bar: 50µm. (ED performed A-D, NLC performed G)
In addition to epithelial cell labeling there was extensive non-epithelial Notch lineage marking with both N1Cre;ROSA-Tom and N2Cre;ROSA-Tom (Fig 2-1F, arrowheads). Histological analysis of tissue sections from the CBF:H2B-Venus reporter mouse demonstrated that the majority of Venus-positive stromal cells are PECAM-1-expressing endothelial cells and α-smooth muscle actin-expressing smooth muscle cells (Fig 2-1G).

**Notch regulation of LGR5 stem cells**

To test the function of Notch1 and Notch2 to regulate antral epithelial cell homeostasis we first examined proliferation after treatment with either inhibitory antibodies that selectively target Notch1 and Notch2 or the gamma-secretase inhibitor DBZ (a pan-Notch inhibitor). Analysis of EdU incorporation showed that cellular proliferation was reduced with combined αN1+αN2 treatment to a similar extent as DBZ (Fig 2-2A-H). Treatment with αN1 or αN2 alone significantly reduced the number of proliferating cells, but to a lesser extent than combined receptor blockade (Fig. 2-2D-H). Analysis at 2 and 4 weeks after αN1 or αN2 treatment demonstrated normal proliferation after the antibody treatment was discontinued (Fig 2-2H). However, morbidity of the αN1+αN2 group prevented analysis of this group at these later time points.

To directly investigate LGR5 stem cells, we treated Lgr5-GFP mice with αN1 and/or αN2 and analyzed stem cell proliferation via co-immunostaining for GFP and Ki67 (Fig 2-2I, arrow). Notch1 and/or Notch2 inhibition reduced LGR5 stem cell proliferation, with αN1+αN2 having the most substantial effect (Fig 2-2J), which was
Figure 2-2. Notch1 and Notch2 regulate antral cell proliferation. (A,B,D-G)
Proliferating cells were detected in (A) vehicle, (B) DBZ, (D) Gd antibody control (αGd),
(E) αN1+αN2, (F) αN1, and (G) αN2 tissues by staining for EdU (green) with DAPI
(red). (C, H) Morphometric quantification of EdU cells (mean ± SEM; n=5-7 mice). (I, J)
Proliferating LGR5 stem cells were visualized in treated Lgr5-GFP mice by GFP (green) and
Ki67 (red) co-staining (arrow) with DAPI (blue) and quantified by morphometrics
(mean ± SEM; n=5-7 mice; 40-100 glands/mouse). *P<0.05, **P<0.01, ***P<0.001,
****P<0.0001 vs. vehicle using Student’s t-test or vs. αGd using 1-way ANOVA. Scale
bar: 50µm (A,B,D-G) or 25µm (I).
similar to our previous analysis of pan-Notch inhibition with DBZ\textsuperscript{9}. The similarity of combined N1 and N2 receptor blockade to pan-Notch inhibition suggests that Notch1 and Notch2 are the key receptors mediating Notch effects on stem cell proliferation.

We next analyzed cell death by staining for the apoptotic marker cleaved caspase 3. Apoptotic cells are normally rare in the antral epithelium. Notch inhibition with DBZ or receptor blockade was observed to increase the number of caspase-positive cells, with apoptotic cells predominantly localized in the gland base (Fig 2-3). As with the proliferation findings, $\alpha$N1+$\alpha$N2 treatment was comparable to DBZ, with more modest effects observed after treatment with antibodies targeting individual receptors (Fig 2-3C-D).

*Notch regulates antral organoid growth*

We established organoids to test whether the Notch effects are intrinsic to the epithelial cells. Organoids established from the *CBF:H2B-Venus* reporter mouse showed extensive Venus labeling, demonstrating active Notch signaling in organoid culture (Fig 2-4A). Gene expression analysis showed that, similar to what was observed in gastric glands, Notch1 and Notch2 were the primary Notch receptors expressed in antral organoids (Fig 2-4B). To investigate Notch function we treated organoids with the pan-Notch inhibitor DAPT and observed reduced overall growth (Fig 2-4C-E). Reduced growth was also observed after receptor targeting, with combined N1+N2 inhibition similar to pan-Notch inhibition, and individual receptor targeting showing a larger role for Notch1 to regulate growth (Fig 2-4F-J). These findings demonstrate that intrinsic Notch signaling is required for antral organoid growth.
**Figure 2-3. Enhanced apoptosis after Notch inhibition.** (A,B, E-H) Apoptosis was measured by co-staining for cleaved caspase-3 (CC3) (green) and E-cadherin (Ecad) (red) with DAPI (blue) in (A) vehicle, (B) DBZ, (E) αGd, (F) αN1+αN2, (G) αN1, and (H) αN2-treated mice. (C,D) Morphometric quantification of apoptotic CC3/E-cad co-stained cells (arrows) (mean ± SEM; n=5-7 mice). Scale bar: 20µm.
Figure 2-4. Notch1 and Notch2 regulate mouse antral organoid growth. (A) Cells undergoing Notch signaling (green) were observed in antral organoids established from the CBF:H2B-Venus Notch reporter mouse strain. (B) Gene expression of Notch receptors determined by qRT-PCR analysis of antral organoids (mean ± SEM n=3 independent organoid lines). ND = Not detected. Morphology (C,D,F-I) and quantitative measure of organoid size (E,J) following 5 days of treatment with (C) vehicle, (D) DAPT, (F) αGd, (G) αN1+αN2, (H) αN1, or (I) αN2 (mean ± SEM; n=3 lines). Scale bar: 50µm (A) or 250µm (C,D,F-I).
Notch1 and Notch2 regulate antral epithelial cell differentiation

We previously showed that Notch inhibition was associated with increased antral cell differentiation\textsuperscript{9}. Thus, we analyzed the role of Notch1 and Notch2 for the major antral cell lineages: enteroendocrine, surface mucous and deep mucous cells. Analysis of enteroendocrine cells showed increased numbers of gastrin-expressing cells and increased abundance of \textit{Gast} and \textit{Chga} mRNAs with Notch inhibition (Fig 2-5). Similar effects were observed after treatment with DBZ (Fig 2-5A-E) and $\alpha$N1+$\alpha$N2, but not with $\alpha$N1 or $\alpha$N2 treatment, suggesting that these two receptors are fully redundant for this function (Fig 2-5F-L). In accordance with the \textit{in vivo} findings, increased \textit{Gast} mRNA was observed in organoids after DAPT or $\alpha$N1+$\alpha$N2 treatment (Fig 2-5M,N).

Similarly, analysis of the surface mucous cell marker CLCA1 showed expansion of this cell population after DBZ (Fig 2-6A-C) or combined $\alpha$N1+$\alpha$N2 treatment, but not after individual receptor blockade (Fig 2-6D-H). Consistent with the marker expression data, ultrastructural analysis showed a marked expansion of surface mucous cells (Supplementary Fig 2-2). Electron microscopy demonstrated increased numbers of granules in these cells after DBZ treatment (Fig 2-6I,J).

Analysis of H&E stained tissues suggested increased mucous cells at the gland base with DBZ or inhibition of both receptors (Supplementary Fig 2-3). Accordingly, GSII-lectin staining showed an apparent expansion of this cell lineage after DBZ or $\alpha$N1+$\alpha$N2 treatment (Fig 2-7A,B,E-H), which was confirmed by increased trefoil factor 2 ($\textit{Tff2}$) mRNA abundance (Fig 2-7C,I). Surprisingly, further analysis of these cells also showed increased staining for the chief cell marker gastric intrinsic factor (GIF), which was confirmed by increased \textit{Gif} mRNA (Fig 2-7D,J). High-powered confocal analysis
Figure 2-5. Notch1 and Notch2 regulate antral endocrine cell differentiation. (A,B, F-I) Gastrin immunostaining (green) of paraffin sections from mice treated with (A) vehicle, (B) DBZ, (F) αGd, (G) αN1+αN2, (H) αN1, and (I) αN2 with DAPI (red). (C,J) Gastrin cells were quantitated by morphometrics (mean ± SEM, n=4-5). Gene expression analysis of gastrin (Gast) and chromogranin A (Chga) in (D,E,K,L) full-thickness tissue or (M,N) organoids (mean ± SEM; n=5-7 mice or 3 independent organoid lines). Scale bar: 50µm.
Figure 2-6. Notch1 and Notch2 regulate surface mucous cell differentiation. (A, B, D-G) Immunostaining for the surface mucous cell marker CLCA1 in (A) vehicle, (B) DBZ, (D) αGd, (E) αN1+αN2, (F) αN1, and (G) αN2-treated mice. (C, H) qRT-PCR analysis of Clca1 gene expression in antral tissue (mean ± SEM; n=5-7 mice). (I, J) Transmission electron microscope images of surface mucous cells from (I) vehicle or (J) DBZ-treated mice. Scale bar: 50µm (A,B,D-G) or 2µm (I,J).
Figure 2-7. Notch inhibition remolds cells at the antral gland base. (A,B, E-H) Co-staining with GSll lectin (green) and an anti-gastric intrinsic factor (GIF) antibody (red) with DAPI (blue) on (A) vehicle, (B) DBZ, (E) αGd, (F) αN1+αN2, (G) αN1, and (H) αN2-treated mice. (C,D I-L) Gene expression analysis of trefoil factor 2 (Tff2) and gastric intrinsic factor (Gif) by qRT-PCR analysis of full-thickness tissue or organoids (mean ± SEM; n=4-7 mice). (M,N) Transmission electron microscope images of gland base cells from (M) vehicle or (N) DBZ-treated mice. Scale bar: 50µm (A,B,E-H) or 2µm (M,N).
showed GSII and Gif co-labeled cells at the gland base, primarily localized in separate granules (Supplementary Fig 2-4). Ultrastructural analysis showed a marked increase in both secretory granule number and granule size in cells at the gland base after Notch inhibition (Fig 2-7M,N and Supplementary Fig 2-2). Consistent with the in vivo data, increased Gif expression was also observed in DAPT or αN1+αN2 treated antral organoids (Fig 2-7K,L).

To better understand the cellular remodeling observed in the Notch-inhibited antrum we examined markers associated with the gastric corpus and intestine. Increased immunostaining and gene expression was observed for the corpus ECL cell marker histidine decarboxylase (HDC; Fig 2-8A-C), however there was no change in expression of the corpus parietal cell marker H,K-ATPase (data not shown). We also observed increased expression of markers of intestinal cells, including goblet cells (Muc2 and Tff3) and Paneth cells (Mmp7, Cryptdin, Reg3γ) in the DBZ-treated antrum (Fig 2-8D-J). However, similar to the corpus markers, the increase in intestinal markers was not associated with a general intestinalization as the DBZ-treated antrum did not increase expression of the intestinal markers Cdx2, Vil1, Cck or Lyz1 (data not shown) and continued to express gastric specific markers (Tff2 and Gif) and

Together these findings suggest that global Notch inhibition with DBZ or combined αN1+αN2 treatment results in a generalized increase in cellular differentiation. However, dual receptor blockade is required for the differentiation effect, suggesting that Notch1 and Notch2 function together during differentiation.
Figure 2-8. Increased expression of gastric corpus and intestinal cell markers in DBZ-treated antral tissue. (A-C) Immunostaining and gene expression analysis of histidine decarboxylase (HDC). (D-F) Immunostaining and gene expression analysis of Muc2. (G-J) Gene expression analysis of trefoil factor 3 (Tff3), matrix metalloproteinase-7 (Mmp7), cryptdin, and regenerating islet-derived 3 gamma (Reg3γ) in antral RNA isolated from vehicle or DBZ-treated mice (mean ± SEM; n=3-6 mice).
**Notch is necessary for growth of human gastric organoids**

We took advantage of the design feature of the inhibitory antibodies to target both mouse and human Notch1 and Notch2 to test the regulation of human gastric stem cells *in vitro* in organoids derived from human gastric glands. We first determined which Notch receptors were expressed by qRT-PCR, showing that similar to our findings in mouse, Notch1 and Notch2 were the predominant receptors expressed in human antral tissue and organoids (Fig 2-9A,B and Supplementary Fig 2-5,2-6, Supplementary Table 2-3).

We confirmed that intrinsic Notch signaling was required for growth of human organoids, with reduced organoid size observed after addition of DAPT to the culture media (Fig 2-9C-E). Treatment with \( \alpha N1+\alpha N2, \alpha N1 \) or \( \alpha N2 \) also reduced growth, with a pattern remarkably similar to what we observed in mouse organoids. These findings suggest that Notch1 and Notch2 together regulate human antral organoid growth and that the mouse serves as a valid model to study human gastric epithelial stem cell function.

**2.5: DISCUSSION**

Here we report that Notch signaling regulates gastric antral epithelial cell homeostasis through the Notch1 and Notch2 receptors. Inhibition of both Notch1 and Notch2 signaling mimics effects on proliferation and differentiation observed with global Notch inhibition with DBZ treatment. Notch signaling in antral LGR5 stem cells was shown by Notch reporter expression and lineage tracing studies. We also showed that Notch signaling is crucial for proliferation of gastric LGR5 stem cells, with both Notch1
Figure 2-9. Human antral organoid growth is regulated by Notch1 and Notch2. (A, B) Notch receptor mRNA abundance was measured by qRT-PCR in (A) full-thickness human tissue or (B) antral organoids (mean ± SEM, n=3 patients or patient-derived organoid lines). See Supplementary Figures 2-5 and 2-6, and Supplementary Table 2-3 for detailed information on human tissues. Morphology (C,D,F-I) and quantitative measure of organoid size (E,J) following treatment with (C) vehicle, (D) DAPT, (F) αGd, (G) αN1+αN2, (H) αN1, or (I) αN2 (mean ± SEM, n=3 independent patient-derived organoid lines). Scale bar: 250µm. ND = Not detected.
and Notch2 functioning to promote stem cell proliferation. Our findings suggest that Notch1 is the primary receptor in LGR5 stem cells as we observed full lineage stripes with N1Cre; ROSA-Tom mice but not with N2Cre; ROSA-Tom mice. In addition αN1 was more effective than αN2 in inhibiting organoid growth. Further studies will be needed to understand how Notch1 and Notch2 regulate transit amplifying progenitors versus stem cells.

Our finding that Notch signaling stimulates gastric stem and progenitor cell proliferation is consistent with previous reports associating Notch pathway activation with gastric tumorigenesis. Upregulation of Notch pathway components has been reported in human gastric cancer, including Notch1, Notch2, Dll4, and Hes1\textsuperscript{10,11,23}, with Notch1 being associated with diffuse type and Jagged1 being overexpressed in both diffuse and poorly differentiated type cancers\textsuperscript{24}. Consistent with a potential role for Notch in promoting gastric tumorigenesis, genetic mouse models of Notch overexpression in the stomach induced hyperplastic polyps\textsuperscript{8,9}.

We further demonstrate that Notch1 and Notch2 regulate epithelial cell differentiation. Inhibition of both Notch1 and Notch2 signaling resulted in significant increases in all differentiated lineages, with increases in endocrine, surface mucous and deep mucous cells, similar to what is seen with global Notch inhibition. Our observation that individual inhibition of Notch1 or Notch2 did not broadly change differentiated cell types suggests that these receptors generally have redundant roles in differentiation. Notch regulation of differentiation in the stomach differs from the action of Notch in the intestine, where it has been well established that pathway inhibition induces a cell fate switch leading to secretory cell hyperplasia\textsuperscript{3,4,6}. Thus, while Notch signaling in the
intestine promotes enterocyte differentiation, Notch signaling in the stomach appears
to generally repress differentiation of all lineages\textsuperscript{9}. It is important to note however that in contrast to the intestine, the adult stomach only contains secretory cells.

Interestingly, we observed that Notch inhibition resulted in marked cellular remodeling. Cells at the surface exhibited an increased number of secretory granules to accompany the observed increases in immunostaining and gene expression of surface mucous cell markers. Tissue remodeling in the gland base was characterized by increased density and size of secretory granules and upregulation of intrinsic factor immunostaining. We also observed an increase in expression of selected intestinal and corpus cell markers, although the expression pattern did not suggest conversion of the antrum to these other gastrointestinal tissues. Further studies are needed to understand the role Notch plays in maintaining antral cell regional identity.

Cells that co-express intrinsic factor and mucous cell markers, such as TFF2, have been previously described in both immature and adult stomach. In mouse, co-expressing cells are observed throughout the immature newborn stomach, including the proximal corpus and distal antral regions\textsuperscript{20}. Moreover, in adult human and mouse stomach, co-expressing cells have been observed in adult corpus as a metaplastic cell lineage termed spasmolytic polypeptide-expressing metaplasia (SPEM)\textsuperscript{25}. SPEM is induced in response to loss of parietal cells and has been proposed to be a precursor to gastric cancer\textsuperscript{26–28}. How the remodeled antral cells in the Notch-inhibited antrum relate to these previously described cell types will be an interesting future question.

The development of methods to grow epithelial organoids from primary mouse and human tissue is a powerful approach to study antral stem cells\textsuperscript{1,22,29,30}. Either
complete Notch blockade with GSI, or specific receptor targeting with αN1 or αN2 inhibitory antibodies had similar effects of reducing overall organoid growth, with Notch1 playing a more significant role than Notch2. This finding agrees with our data showing active Notch1 signaling in antral LGR5 stem cells. Furthermore these studies showed that Notch signaling is intrinsic to the gastric epithelium, with signaling required for organoid growth in culture. In addition, we observed that Notch regulates organoid differentiation, with upregulation of zymogenic and endocrine cell marker expression after Notch inhibition with DAPT or combined αN1+αN2 treatment, similar to what we observed in vivo.

Our finding that Notch1 and Notch2 receptor signaling regulates human stomach epithelium has therapeutic implications. Notch pathway dysregulation is associated with the development of several cancers and thus this pathway is a prime target of therapeutic significance. The use of pan-Notch inhibitors such as GSIs for Notch-related pathologies has been of clinical relevance for years; however gastrointestinal toxicity severely limits their use. Thus more specific pathway targets are being explored. In particular, the development of humanized inhibitory antibodies against either Notch1 or Notch2 might allow for dose-dependent inhibition of each receptor while avoiding the side effects seen with global Notch inhibition. However, our findings predict that targeting Notch1 or Notch2 to treat cancer might be associated with disruption of gastric epithelial cell homeostasis and thus gastric cellular changes should be considered in patients undergoing targeted Notch inhibition.
2.6: ACKNOWLEDGMENTS AND FUNDING

We thank Dr. Thaddeus Stappenbeck for L-WRN cells, Dr. John Williams and Bradley Nelson for assistance with electron microscopy, Dr. Spyros Artavanis-Tsakonas for use of the N1Cre and N2Cre mice and Christopher Altheim for generating L-WRN conditioned media. GBG was supported by NIH T-32-GM0008322 and NIH T32-DK094775, ESD was supported by NIH F32-DK093349, and ESD and PHD were supported by NIH T32-HD007505. The research was funded by an award from Biogen to AL, by NIH P01-DK06041 and NCI P50-CA130810 project awards to LCS and Core support from the Michigan Gastrointestinal Research Center Grant NIH P30-DK34933 and the University of Michigan Cancer Center Support Grant NCI P30-CA6592.

COMPETING INTERESTS

CWS is an employee of Genentech Inc. and owns shares of Roche. All other authors state that they have no conflicts of interest.
**Supplementary Table 2-1. Antibodies and lectins used for histological analysis**

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*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). Staining in cryo sections did not use antigen retrieval.*
## Supplementary Table 2-2. Oligonucleotide sequences used for qRT-PCR

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*Sequences designed to amplify the human gene; all other primers amplify the mouse gene.*
Supplementary Table 2-3. List of patient information for human tissues

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Supplementary Figure 2-1. Positive control for Notch4 qRT-PCR assay. mRNA abundance measured in (A) pancreatic tissue (mouse) or (B) fat tissue (human) (mean ± SEM of technical replicates, n=1).
Supplementary Figure 2-2. Altered cellular morphology with Notch inhibition. Semi-thin sections of antral gland base and surface regions from (A,C) vehicle or (B,D) DBZ-treated mice stained with toluidine blue. Scale = 10μm.
Supplementary Figure 2-3. Antral gland histology after Notch inhibition. Paraffin sections stained with haematoxilin and eosin (H&E) from (A) vehicle, (B) DBZ, (C) αGd, (D) αN1+αN2, (E) αN1, or (F) αN2-treated mice. Scale bar: 50 µm.
Supplementary Figure 2-4. Notch inhibition leads to increased GSII/GIF co-staining at the antral gland base. Confocal microscopy of paraffin tissue sections co-stained for GSII and GIF in (A) vehicle and (B) DBZ-treated mice. Occasional co-stained cells were detected in vehicle treated mice at the gland base. There was a marked increase in co-stained cells after Notch inhibition. Separate channels for GSII (A', B') and GIF (A'', B'') are shown. Although the majority of granules appear to be specific for GSII or GIF, there were also some co-stained granules (arrowheads). Scale bar: 10µm.
Supplementary Figure 2-5. Notch receptor expression in human gastric tissue. Notch receptor mRNA abundance was measured in (A) full-thickness antral tissue samples and (B) established organoids. Patient samples are listed in Supplementary 2-3. Shown are results of qRT-PCR analysis in each patient tissue sample or organoid line as marked (mean ± SEM of technical replicates). ND = Not detected.
Supplementary Figure 2-6. Histology of human gastric tissue samples used in this manuscript confirms antral tissue identity. Paraffin sections from individual human samples were stained with haematoxilin and eosin (H&E). Scale = 50 µm.
REFERENCES


