INSIGHTS INTO THE DEVELOPMENT OF GASTROINTESTINAL BRUNNER'S GLANDS: CRITICAL STEM CELLS AND DIFFERENTIATION FACTORS

by

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A THESIS

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Gastrointestinal Brunner's Glands are composed of secretory glandular epithelium and are located at the junction between the pylorus of the stomach and the duodenum of the small intestine. Brunner's Glands function by secreting a mucous-rich substance containing sodium bicarbonate and other important proteins in order to neutralize stomach acid and prevent damage to the intestinal epithelium. Prior research has shown that Brunner's Glands develop after birth, but little is known about the molecular mechanisms that control their development. Tumors of the in Brunner's Glands are known to occur in humans and understanding their development may assist in understanding how cancer and other pathologies in this gland arises. The aim of this study is to evaluate the stem cells and proteins that contribute to the development of the gland by using the laboratory mouse as our model organism.

A stem cell marker known as *Lrig1* is a regulator of intestinal stem cell development and by using a technique known as "developmental lineage tracing," we have shown that *Lrig1* expressing stem cells contribute to the development of the Brunner's Gland. Developmental lineage tracing was also used in order to test for the

contribution of *Villin* to the formation of the Brunner's Gland, and this is another intestinal marker important for differentiation. Our results showed that Brunner's Glands do not express *Villin*. In addition, since Brunner's Glands are secretory glands, we hypothesized that a transcription factor called *SPDEF* is critical for proper development as it is an important protein for secretory gland formation. By analyzing the small intestines of transgenic mice with *SPDEF* mutations at multiple stages of maturity, we find that Brunner's Glands were largely deformed and underdeveloped.

These findings suggest that Brunner's Glands are more closely related to the gastric epithelium, rather than the intestinal epithelium as has been previously postulated. Overall, the results of this study provide insight into the specific progenitor cells that contribute developmentally to the Brunner's Gland**,** and shed light on the molecular mechanisms important for proper formation.

Acknowledgements

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Introduction

Brunner's glands are unique to mammalian species and are located in the proximal small intestine. The Brunner's gland is labeled as a secretory gland because has mucus-rich alkaline secretions containing sodium bicarbonate and other factors. These secretions are used to neutralize stomach acid, chyme, and aid in the regulation of ion and fluid transport (Collaco et al., 2013). Without this gland, it is possible that ulceration could occur in the intestine, as highly acidic materials passing from the stomach can damage the intestinal lumen. In addition to protection, Brunner's glands assist in lubrication of intestinal walls, assisting in the travel of materials smoothly down the intestine during digestion.

Although Brunner's Gland adenomas are rare, they can result in gastrointestinal hemorrhage and duodenal obstruction (Gao et al., 2006). While this is unfortunate enough, another study found a strong correlation between Brunner's gland adenomas and concurrent *Helicobacter pylori* (*H. pylori*) infections (Kovacevic et al., 2001). Chronic inflammation arises from *H. pylori* infections and this significantly escalates the risk of developing gastric and intestinal cancer, and duodenal and gastric ulcer disease (Wroblewski et al., 2011). Brunner's gland adenomas may start as a benign pathology, but they can lead to more serious complications that may be malignant or life-threatening. Therefore, it is vital to study the role of all proteins and stem cells that contribute to proper Brunner's gland development in order to form a more comprehensive understanding of the etiology of Brunner's gland pathologies.

To date, there has been a great deal of research completed on the participation of ion transport and production of secreted factors in the Brunner's gland, but little is

known about the factors that influence their development. In biology, structure dictates function and the proper function of this gland is a result of highly regulated developmental processes. Defects or deficiencies in structure or function are often a direct result of disruptions during the developmental processes. In this study, we used transgenic mice with mutations in intestinal development pathways, to determine their contributions to the development of the Brunner's gland.

Why use Mus musculus as the model organism?

It is normal to wonder how studying the intestine of mice is going to help us learn about the intestine of humans, however, the laboratory mouse or *mus musculus* is one of the most prominent model organisms used to research human disease progression and development. Although mice may not seem similar to us in size or appearance, humans and mice are very similar genetically. Mice are able to reproduce and mature very quickly, and this is advantageous in mouse husbandry where one can create a required mutant mouse for an experiment in a timely and inexpensive manner. Mice mature and can reproduce at six weeks of age, and there exists a large catalog of transgenic mice with different genotypes that can be ordered from other labs and companies for use in research. The model organism does have some limitations, as not all findings in mice will translate directly to humans, but research in mice can provide a strong foundation to understanding biological processes in humans.

Small Intestine Anatomy

Before further discussion of the Brunner's gland development, it is important to understand the context of the organ that it is located in. The small intestine is divided

into three sections known as the duodenum, jejunum and ileum. Its main functions include the secretion of enzymes and digestive aids, propulsion and chemical digestion of food, and absorption of nutrients and fluids (Moore, 2014).

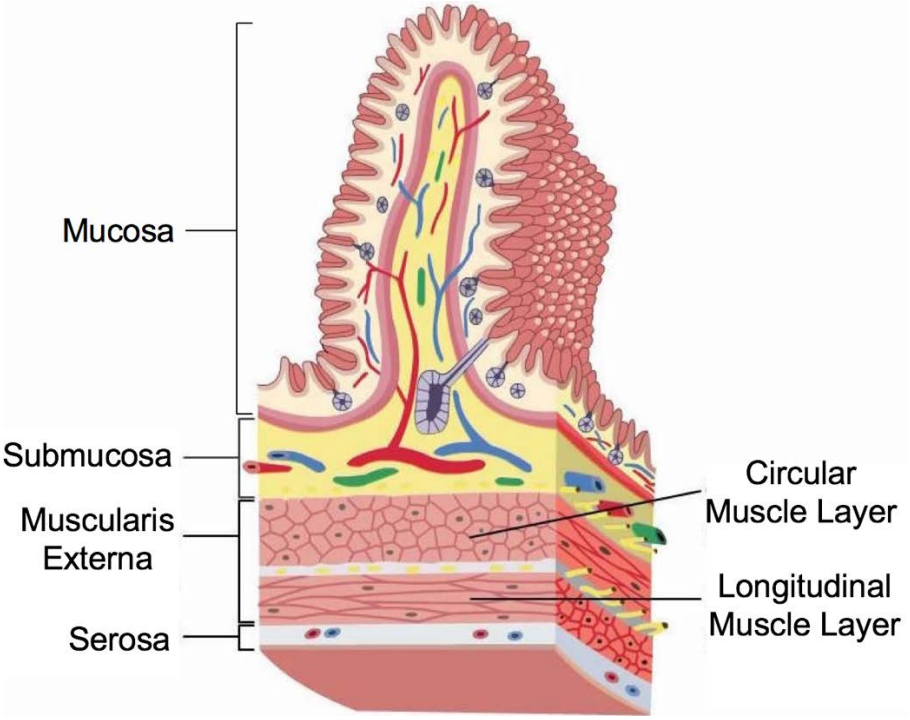

Figure 1: The Layers of the Small Intestine

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The area of interest for this study is the duodenum and this is comprised of four main layers that include the mucosa, submucosa, muscularis externa, and serosa (Figure 1). The serosa is the most superficial (or outermost) layer of the small intestine and secretes serous fluids which act as lubricant to reduce friction with neighboring thoracic structures during intestinal muscle contractions (Moore, 2014). The muscularis externa is deep to the serosa, and includes the circular and longitudinal muscle layers which contract in order to propel food down the intestine (Moore, 2014). The submucosa is located deep to the muscularis externa and contains the blood vessels, nerves, and

intestinal glands which support the mucosa. The mucosa is the innermost layer that faces the lumen of the intestine, and consists of crypts and villi that allow for food to travel down its surface while absorbing nutrients and fluids of anything that passes through (Moore, 2014). The Brunner's Gland is located specifically in the submucosal layer of the proximal duodenum, distal to the pylorus of the stomach (Figure 2). It is a comma-shaped gland and extends for variable distances distally in the duodenum, but are normally 2-5 millimeters long in the mouse (Obuoforibo et al., 1978).

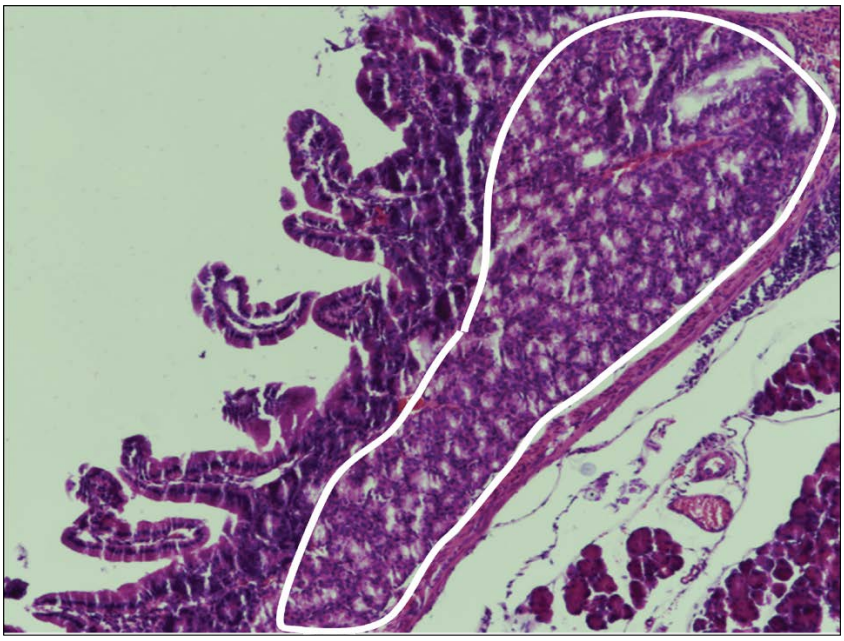

Figure 2: Hematoxylin and Eosin Staining of a Wildtype Brunner's Gland

The Brunner's gland is outlined in white, and is a comma-shaped gland in the submucosal layer of the proximal intestine. (Parappilly et al., unpublished)

Brunner's Gland Function

Brunner's glands are composed of secretory glandular epithelium that contain serous cells and branching tubules of variable lengths. The ducts of individual glands drain into the the base of crypts in the duodenum (Treasure, 1978, Figure 2). The main

secretory unit found in the Brunner's gland secretions are mucin glycoproteins which form a viscous barrier that covers the mucosa of the small intestine. This barrier provides resistance to substances such as acid, pepsin, NaCl, bile, and ethanol, but is still permeable enough for ions and small molecules to pass through to the intestinal epithelium (Mcqueen et al., 1983; Bell et al., 1985). No direct evidence has been found that shows the bicarbonate production originates in the gland, but the gland does contain bicarbonate transport proteins (Collaco et al., 2013) allowing for the transfer of this alkaline substance to the lumen of the duodenum. Not only does the Brunner's gland secrete glycoproteins and bicarbonate, but it also secretes many other factors, most notably epidermal growth factor, insulin-like growth factor 1, and trefoil peptides. The function of epidermal growth factor (EGF) is to inhibit gastric acid secretion (Bower et al., 1975). The functions of insulin-like growth factor 1(ILGF1) include promoting immunological defense, cellular proliferation, and differentiation (Ryan et al., 1993). Trefoil peptides are involved in the oligomerization process of the mucin glycoprotein molecules (Krause, 2000), and this assists the formation of the protective barrier on the luminal side of the intestine.

It was originally hypothesized that the Brunner's Gland was important only for the neutralization of stomach acid and protection of the intestinal wall. We now know its secretion has many more components than originally discovered and these have numerous functions important for intestinal homeostasis, as it creates an environment that is ideal for intestinal enzyme efficiency (Krause, 2000).

What is known about Brunner's gland development?

Much of the research completed on the Brunner's gland has focused on the physical growth of the gland and its function as opposed to the molecular mechanisms that contribute to its development. Previous research has shown that Brunner's glands in mice attain their adult form 21 days after birth (Obuoforibo et al., 1977). Little is known about the factors that control Brunner's gland development but there has been a fair amount of research on the development of other secretory cells in the intestine. From this, we know that proper development depends on many proteins, but we have yet an incomplete understanding of the process.

Through a method called "developmental lineage tracing," research has shown that a protein called *Leucine-rich repeats and immunoglobulin-like domains 1* (*Lrig1)* is an intestinal stem cell marker that is important for homeostasis and tumor suppression through *ErbB* receptor suppression. (Powell et al., 2012). *ErbB* receptors in the intestine promote proliferation of stem cells which can lead to tumor growth (Roberts et al., 2002). Many cells in the intestine express *Lrig1* and it raises the question whether Brunner's gland cells express *Lrig1* at any point in their development. Another protein known as *Villin* has been shown to be expressed in significant amounts in most intestinal epithelial cells (Wang et al., 2008), but not gastric (stomach) cells. If Brunner's glands express *Villin*, it supports the idea that the gland is of intestinal origin, as opposed to a gastric origin. This study will aim to test the presence of both *Lrig1* and *Villin* expression in the Brunner's gland.

Stem cells in the intestine can give rise to many different lineages, but the greatest distinction is whether or not daughter cells take on an absorptive fate or

secretory fate. The protein *NOTCH* signals stem cells to become absorptive progenitor cells, while the protein *Wnt* signals stem cells to become secretory progenitor cells (Barker, 2014, Figure 3). Since Brunner's glands are secretory cells, *Wnt* signaling is critical for its development. This study did not directly test for the contributions of *Wnt* to Brunner's gland development, but because Brunner's glands are secretory intestinal cells, an assumption was made that *Wnt* starts its signaling cascade for development.

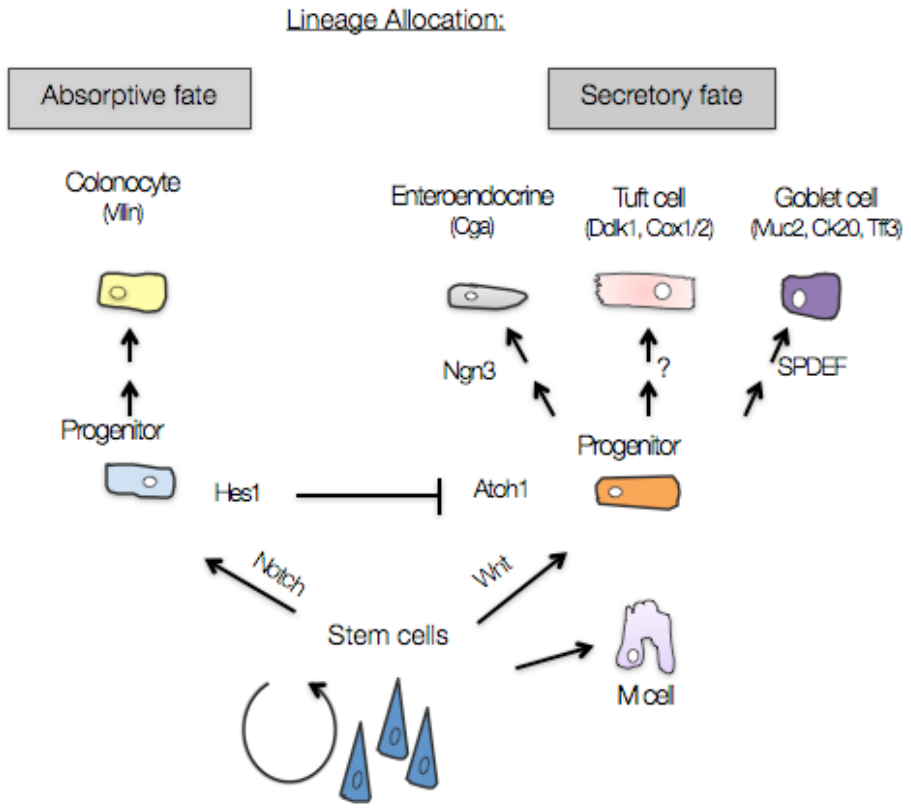

Figure 3: Lineage Allocation in Intestinal Stem Cells

Stem cells in the intestine may take on an absorptive fate or secretory fate depending on particular signals early in differentiation. Signaling by *Notch* protein pushes stem cells toward an absorptive fate and signaling by *Wnt* protein pushes stem cells toward a secretory fate. (Powell Lab – University of Oregon, unpublished)

Brunner's glands are not absorptive cells, but the regulation of lineage allocation between absorptive and secretory fates are highly interrelated. By blocking *Notch* signaling, an upregulation of *Wnt* signaling was observed in intestinal stem cells and this indicates that *Notch* signaling suppresses *Wnt* signaling (Tian et al., 2015). This begs the question whether an inhibition of *Notch* will have any effect on the development of Brunner's glands. Is it possible that an overexpression of *Wnt* signaling could lead to overdeveloped Brunner's glands?

If progenitor cells have been exposed to *Wnt* signaling and have taken a secretory fate, there are numerous other factors that further regulate the type of intestinal secretory cell they will develop into. Enteroendocrine cells, tuft cells, and goblet cells are all secretory cells that originate from secretory progenitor cells. Downstream of the *Wnt* signal in the secretory pathway are the proteins *Atoh1 (Math1)* and *Gfi1*. A previous study found that in mutant *Atoh1* null mice, there is an absence of *Gfi1* expression (Shroyer et al., 2005). *Gfi1* functions downstream of *Atoh1* in the intestinal epithelium, where it selects goblet versus enteroendocrine cell fates among intestinal secretory progenitors (Shroyer et al., 2005). Further downstream of *Gfi1* is a protein called *SAM Pointed Domain ETS Factor* (*SPDEF)*, and it has been found to be sufficient to promote goblet cell differentiation (Noah et al., 2010).

As previously stated, Brunner's glands are secretory cells, and the aim of the study is to evaluate the factors critical for development. Goblet cells, the most similar cell to Brunner's gland cells, function to protect the intestinal epithelium by synthesizing mucin glycoproteins (Specian et al., 1991). Since these two secretory cells are alike, it is possible that they share same of the same signaling cascades.

Using transgenic mice with mutations specific to both absorptive and secretory signaling cascades, we are able to evaluate the contributions of these factors to the development of Brunner's glands, as well as the presence of expression of *Lrig1* and *Villin* through qualitative observation.

Methods

Preparation of Tissues:

This study used wildtype mice for control and transgenic mice of varying genotypes in order to observe any morphological or functional changes to the Brunner's gland. Mice were bred carefully and in sterile conditions in order to minimize possibilities of intestinal diseases or other pathologies that may affect their development or homeostasis. Brunner's glands were observed at multiple developmental stages which include postnatal day 1 (p1), postnatal day 14 (p14), and as adult mice (>6 weeks of age). At desired age, mice were sacrificed humanely under Animal Care Services (ACS) regulations and then dissected for the stomach and small intestine. These organs were then washed in a phosphate-buffered saline solution to rinse them of any contaminants and then cut and flayed into a 4% paraformaldehyde (PFA) solution for preservation. Depending on the experiment, tissues were then immersed overnight in a 30% sucrose solution to be embedded in frozen blocks, or immersed in 70% ethanol solution to be embedded in paraffin wax blocks. Frozen blocks are cut on a cryotome while paraffin blocks were cut on a microtome. These machines allowed for the cutting of 5-10 micrometer sections of tissue which could then be placed on glass slides making them ready for immunohistochemistry, immunofluorescence, and other histological analysis processes.

Histology:

Hematoxylin and Eosin (H&E) staining is one of the most basic and common methods used in histology. Hematoxylin is a violet stain that is basic and positively

charged which allows it to bind to the nucleus of cells where DNA and RNA, which are negatively charged, are located. Eosin is a pinkish-red stain that is acidic and negatively charged which allows it to bind to the cytoplasm, extracellular matrix, and muscle. This stain allows to easily see the morphology of the Brunner's gland under a brightfield microscope, and qualitative observations can be made between wildtype and mutant mice. H&E staining was completed on paraffin sections of wildtype mice, and differentiation pathway mutants including: *SPDEF* knockout mice, *Gfi1* knockout mice, *Atoh1* knockout mice, *Rbpj* knockout mice, as well as *Notch* signal inhibited (DBZ treated) mice.

Alcian Blue-Periodic acid-Schiff (PAS) staining is another common stain used in histology. Alcian blue will stain all acidic mucins a deep blue while PAS will stain neutral mucins a bright magenta, thus making it useful for differentiation of neutral mucins from acidic mucins in paraffin tissue sections under a brightfield microscope. Alcian blue-PAS staining was completed on wildtype and SPDEF knockout paraffin sections.

Antibody Staining (Immunofluorescence and Immunohistochemistry):

Antibody stains were completed on *SPDEF* knockout mice and wildtype mice for control. Antibody staining starts off with slides undergoing antigen retrieval in a citrate buffer so that the protein antigens are exposed and easily bound by antibodies. A specific, primary antibody is then added in order to target the protein of interest and is incubated overnight. The next day a fluorescent secondary antibody is added which binds to the original, primary antibody. This adds a fluorescent marker to the protein of interest on the slides, and can be observed under a fluorescence microscope. Finally, the

slides with tissue are washed in PBS solution containing a stain called 4',6-diamidino-2-phenylindole (DAPI). DAPI is a substance that emits a blue fluorescence, under UV-excitation, and binds to all nuclei in the cells, allowing for further visualization by providing context to the size and shape of the cells. The primary antibodies used in this study were: Trefoil Factor 2 (TFF2), Aquaporin 5 (AQP5), ErbB2, Mucin4 (Muc4), Mucin5AC (Muc5), and Mucin6 (Muc6).

Developmental Lineage Tracing:

To examine the expression of *Lrig1* and *Villin* in the Brunner's gland, a method known as developmental lineage tracing was used. This process allows for the identification of all the progeny of a single cell, by labeling them with a specific marker. Once one "mother" cell is labeled, all of its "daughter" cells inherit this label and this process gives information on the origination of cells. During development, some factors may be expressed early in the life cycle, and may lose the expression of these factors as it matures. Lineage tracing can help us determine whether cells originated from cells expressing these factors or markers.

To test for expression of *Lrig1* in Brunner's glands, lineage tracing was performed by a single injection of tamoxifen (at a dose of 30mg/kg) into p1 *Lrig1-CreERT2/+;R26R-YFP/+* mice. This transgenic mouse contains the CreERT2-recombinase at every cell where *Lrig1* is present. The CreERT2-recombinase is activated by tamoxifen, and when this happens CreERT2 recombines with the nucleus of the cell and expresses Yellow Fluorescent Protein (YFP). At p14, these mice are sacrificed and their Brunner's glands were embedded in paraffin blocks and cut into sections ready to undergo antibody staining. A primary antibody that binds to YFP was

first stained on the sections, and then a fluorescent secondary antibody that binds to the primary antibody was added. Since this tissue was embedded in paraffin, a “tyramide signal amplification (TSA) kit” is necessary in order to amplify the fluorescent signals in the tissue which allows for easier observation during microscopy. The TSA kit functions by using a horseradish peroxidase (HRP) secondary antibody that binds to the primary antibody used. This HRP antibody can then be activated using a tyramide solution and leads to extremely bright reactions in the vicinity of the primary antibody locations, that stand out amongst other signals. After staining, these sections of Brunner’s gland tissue are observed under a fluorescent microscope in order to detect the expression of *Lrig1* through the presence of YFP.

To test for expression of *Villin* in Brunner’s glands, lineage tracing was performed by a single injection of tamoxifen (at a dose of 30mg/kg) into p1 *VillinCreERT2-YFP* mice. This transgenic mouse contains the CreERT2-recombinase at every cell where *Villin* is present. In a similar mechanism as above, tamoxifen activates the CreERT2 recombinase, and YFP is expressed wherever *Villin* is located. At p14, these mice are sacrificed and their duodenums are embedded in frozen blocks, where antibody staining is used to fluorescently mark YFP in the Brunner’s gland tissue. This process did not require the TSA kit; as YFP signals are fairly strong in frozen blocks. The slides were then observed under a fluorescent microscope in order to detect the expression of *Villin* through the presence of YFP in the Brunner’s gland.

Results

We first wanted to examine which proteins are important for Brunner's gland formation. As seen in figure 4, we observed extensive differences in morphology between wildtype Brunner's glands and *SPDEF* null Brunner's glands, based on H&E staining. *SPDEF* null mice exhibit Brunner's glands that are largely underdeveloped and deformed. This provides the first evidence that *SPDEF* is important for Brunner's gland development.

Adult Brunner's Glands SPDEF Knockouts:

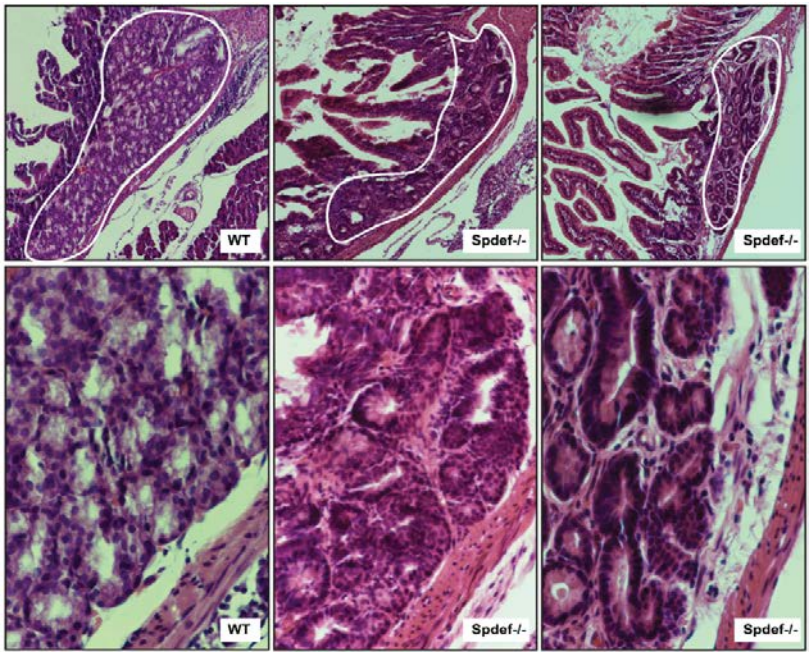

Figure 4: Morphology of Wildtype Brunner's gland vs. SPDEF Knockouts

Hematoxylin and Eosin staining of wildtype Brunner's glands and mice mutant for the intestinal secretory differentiation factor SPDEF. These knockout mice exhibit smaller glands and appear largely deformed. The top row contains images in a 20x objective while the bottom row contains images taken in a 40x objective. (Parappilly et al., unpublished)

We then wanted to examine upstream regulators of intestinal differentiation.

Adult Brunner's Glands Lineage Pathway Mutants:

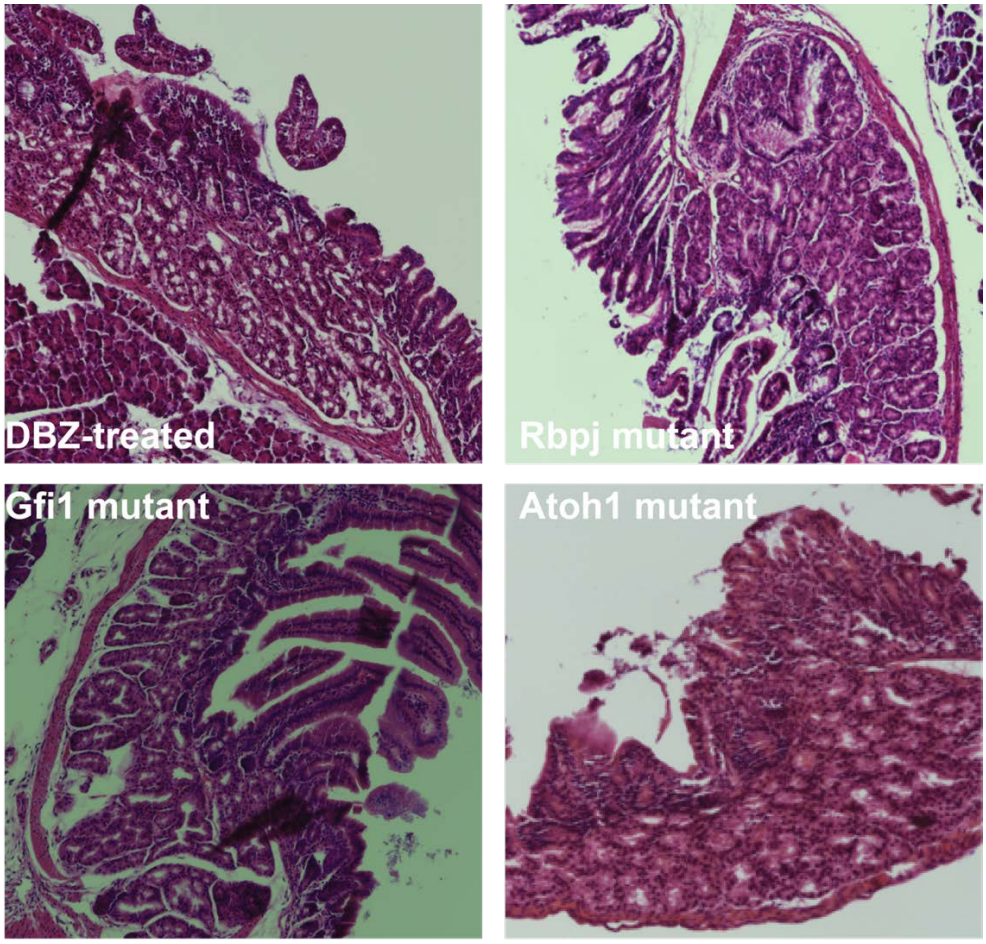

Figure 5: Morphology of Brunner's Glands with Lineage Pathway Knockouts

Hematoxylin and Eosin staining of Brunner's glands with different pathway mutations. DBZ treatment inhibits the expression of *Notch* which controls the absorptive cell pathway. *Rbpj* is also responsible for the expression of *Notch*. *Atoh1* and *Gfi1* knockouts control secretory progenitor cell differentiation. These mutants did not significantly affect Brunner's gland morphology. (Parappilly et al., unpublished)

Rbpj is a co-transcriptional activator factor in *Notch* signaling, and so an upstream *Rbpj* knockout can be used to inhibit *Notch* signaling. *Dibenzazepine (*DBZ) treatment is another method used to inhibit *Notch.* By analyzing morphology of both

Rbpj knockouts, and DBZ treated Brunner's glands, we are able to test whether *Notch* signaling is important for Brunner's gland development. Inhibition of *Notch* signaling showed no significant differences from wildtype Brunner's glands (Figure 5). *Gfi1* and *Atoh1* are protein signals important in formation of goblet cells which are the secretory cell most similar to Brunner's gland cells. However, *Gfi1* knockouts, and *Atoh1* knockouts had no significant effects on Brunner's gland morphology. These findings provide evidence that Brunner's gland development is not strongly dependent on *Notch*, *Gfi1,* or *Atoh1* (Figure 5).

Since *SPDEF* knockouts harbored significant defects in the morphology of the Brunner's gland, we wanted to test whether these mutants led to a loss of function or differences in function of the gland. Alcian blue-PAS staining was used to test for differences between the expression patterns of mucins in the wildtype and mutant Brunner's gland. This can provide further insight into differences in mutant Brunner's glands, as changes in the distribution of expression of the mucins can be indicative of functional differences (Myers, 2010). Brunner's glands retained similar expression of neutral and acidic mucins between wildtype and SPDEF knockout mice (Figure 6).

Alcian Blue-PAS Staining:

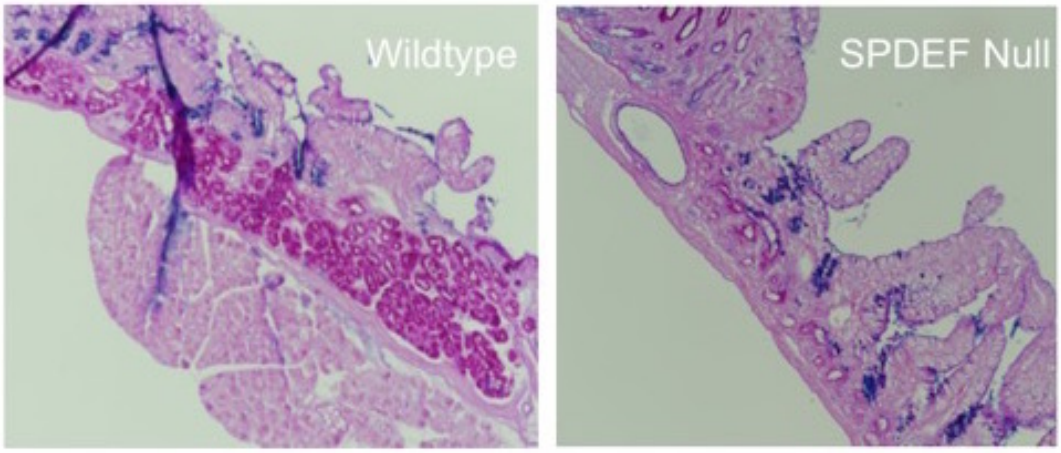

Figure 6: Alcian Blue/PAS Analysis of Wildtype and *SPDEF* Null Brunner's Glands

Alcian blue stains acidic mucins a deep blue, while PAS stains neutral mucins magenta. The expression pattern of alcian blue-PAS remained similar in wildtype and SPDEF null Brunner's glands (Parappilly et al., unpublished)

While it appears that *SPDEF* null Brunner's gland express much fewer neutral mucins (magenta, Figure 6) than compared to the wildtype gland, this is likely due to *SPDEF* knockout Brunner's glands being much smaller than the wildtype. The important observation that we made here is that Brunner's gland cells are magenta colored in both wildtype and *SPDEF* knockout glands. Therefore, the secretions remain similar in pH level between these genotypes.

Although *SPDEF* knockouts secrete mucins of a similar pH, we wanted to test whether there were other functional differences between wildtype and *SPDEF* knockout glands. A series of antibody stains were completed on wildtype and SPDEF null mice to see any differences in protein expression. These antibodies included TFF2, AQP5, ErbB2, Muc4, Muc5AC, and Muc6 (Figures 7 and 8). TFF2 expression is seen in secretory proteins with at least one copy of the trefoil motif in the gastrointestinal

mucosa (Krause, 2000). The Brunner's gland secretes trefoil peptides and so expression should be observed under a fluorescent microscope. Upon examination of the wildtype and mutant tissues, we saw that TFF2 expression remained normal between wildtype and *SPDEF* null Brunner's glands; this is illustrated in Figure 7.

AQP5 is a water channel that has been identified in wildtype Brunner's glands, and is used to provide a pathway for osmotic water movement and electrolyte secretion through the membrane of the gland (Collaco et al., 2013). An AQP5 antibody was stained on wildtype and *SPDEF* knockout mice, in order to look for any expression differences in the gland, which may imply a loss of this function. Importantly, AQP5 expression is lost in *SPDEF* knockout Brunner's glands.

ErbB2 is a protein that is involved in epithelial growth regulation and is normally expressed in crypts and villi of the intestine, and also in the Brunner's gland itself (Wang et al., 2015). An ErbB2 antibody was used on wildtype and *SPDEF* knockout glands, in order to look for differences in expression between the two animals. We found that ErbB2 expression remained similar in wildtype and *SPDEF* knockout mice (Figure 7).

TFF2, ErbB2, Muc6, AQP5 Staining of Adult Brunner's Glands:

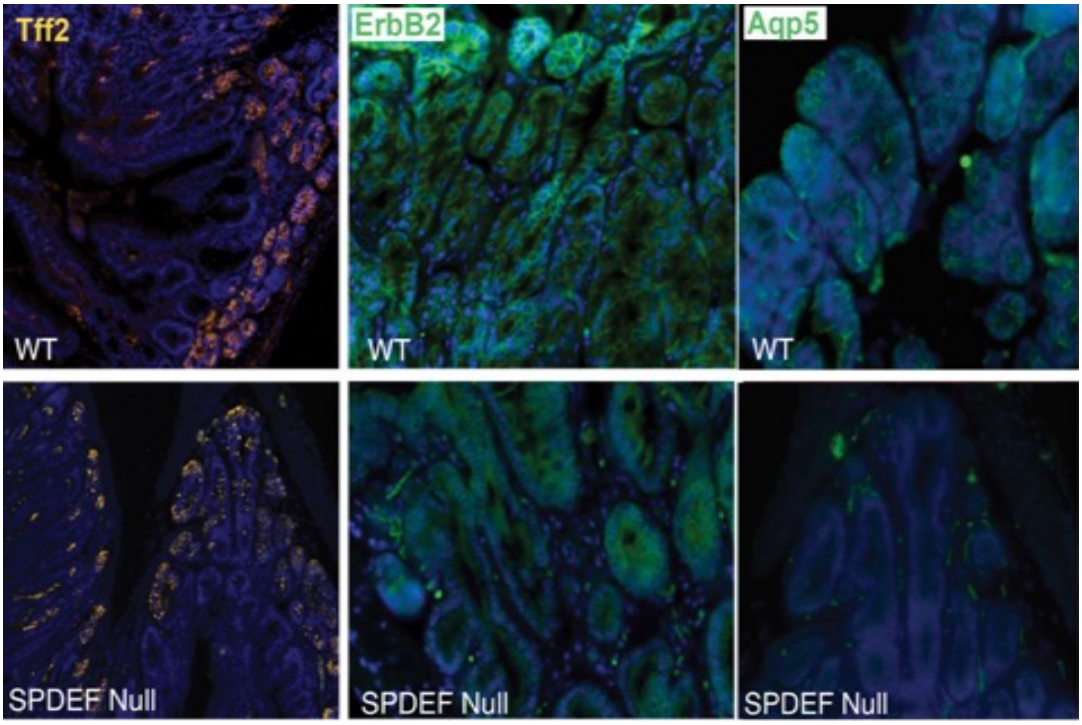

Figure 7: Adult Brunner's Glands Antibody Stains – TFF2, ErbB2, AQP5

TFF2 is expressed in yellow, ErbB2 is expressed in green, AQP5 is expressed in green, and DAPI is expressed in blue. SPDEF null mice exhibit normal expression of TFF2, and ErbB2, but they harbor a defect in AQP5. (Parappilly et al., unpublished)

Three antibody stains in the mucin family were completed on *SPDEF* null and wildtype mice including Muc4, Muc5AC, and Muc6. Muc4 is a mucin protein that is expressed in higher quantities in the colon than compared to the small intestine, and is found to be in colonic goblet cells, but not goblet cells of the small intestine (Rong et al., 2005). Muc5AC is a mucin protein that is localized in the stomach, but not in the intestine (Longman et al., 2000). Muc6 is a mucin protein that is exclusively expressed in Brunner's glands, and not in the rest of the small intestine (Krause, 2000). These antibody stains were completed to look for changes in expression between the mutant mice and wildtype mice, and may provide evidence of their developmental origin.

Muc4, Muc5AC, and Muc6 expression remained similar between wildtype and *SPDEF* knockout mice (Figure 8), and indicates the loss of *SPDEF* does not affect these mucins.

Muc4, Muc5AC, and Muc 6 Staining of Adult Brunner's Glands:

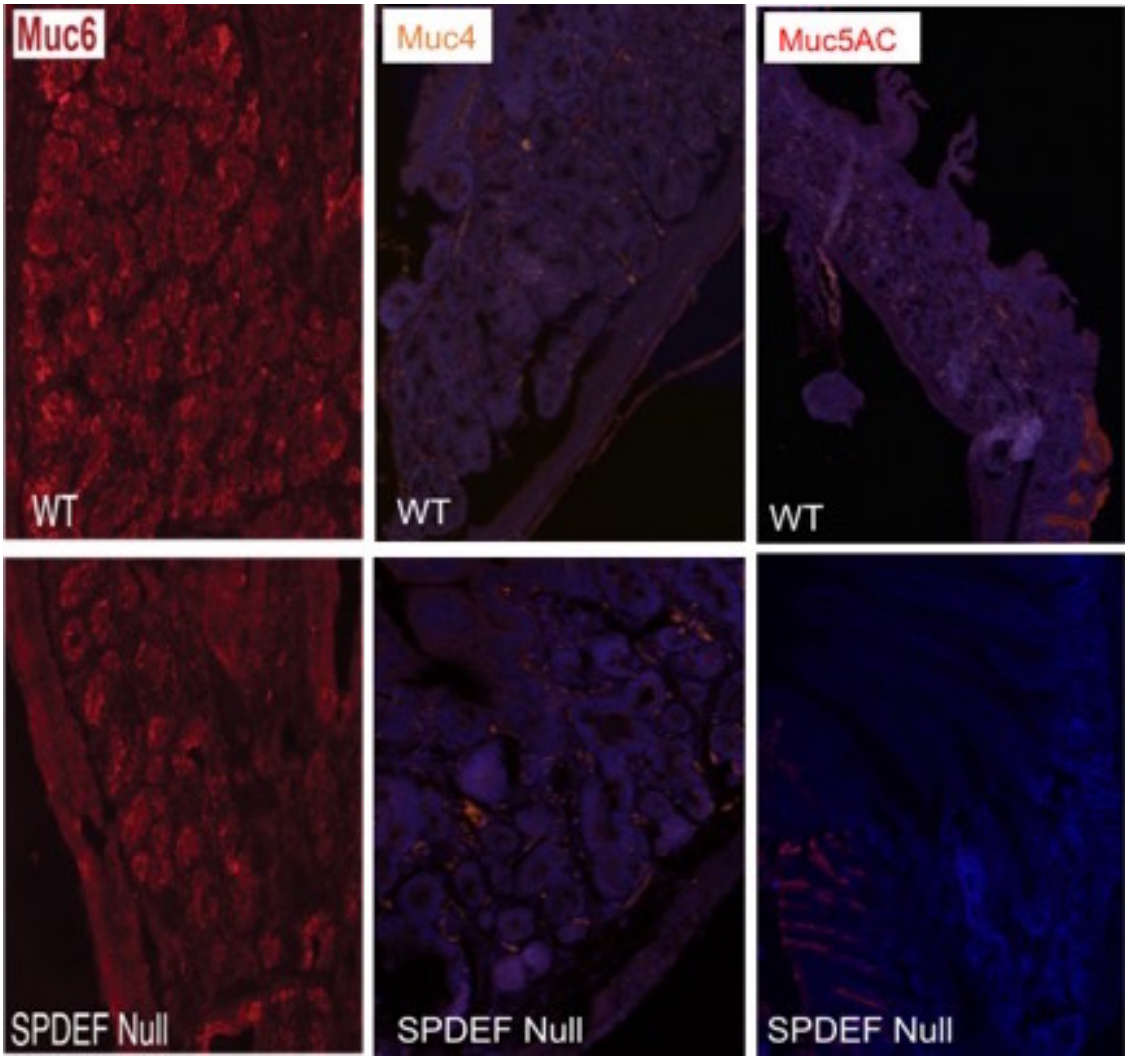

Figure 8: Adult Brunner's Glands Antibody Stains – Muc6, Muc4, Muc5AC

Muc6 is expressed in red, Muc4 is expressed in orange, and Muc5AC is also expressed in red. SPDEF null mice exhibit normal expression of Muc6, Muc4, and Muc5AC. (Parappily et al., unpublished)

Lastly we wanted to test whether Brunner's glands originate from cells that express *Lrig1* or *Villin. Lrig1* is found in many different tissues of the body including the stomach and small intestine, and it functions as a stem cell marker in both of these organs (Powell et al., 2012). Using developmental lineage tracing of *Lrig1Cre-YFP/+* mice, we detected YFP in the Brunner's gland cells, supporting the idea that they came from *Lrig1*-expressing progenitor cells (Figure 9). *Villin* is expressed in all intestinal epithelial cells, but it is not expressed in the Brunner's gland, and so we wanted to examine if *Villin*-expressing progenitor cells could give rise to the Brunner's gland cells. Using a similar approach, we examined lineage-traced *VillinCre-YFP* mice, and observed that Brunner's gland cells do not arise form *Villin* expressing progenitor cells (absence of YFP in the gland, Figure 10).

Lineage Tracing of Lrig1 Expression in Brunner's Glands:

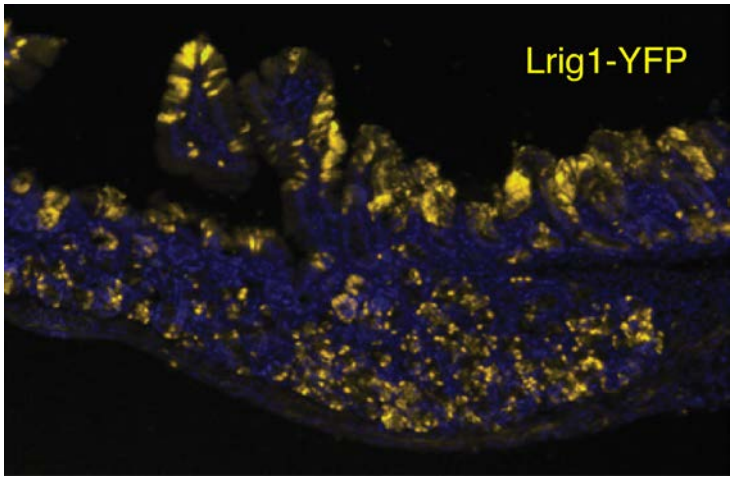

Figure 9: Lineage Tracing of Lrig1-YFP Brunner's Glands

Cells arising from *Lrig1* expressing progenitor cells are expressed in yellow, while DAPI is expressed in blue. Lineage tracing was performed from p1-p14. (Parappily et al., unpublished)

Lineage Tracing of Villin Expression in Brunner's Glands:

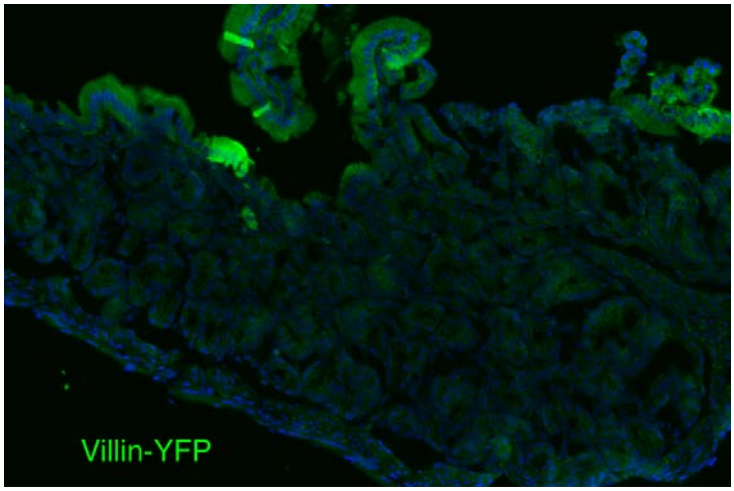

Figure 10: Lineage Tracing of Villin-YFP Brunner's Glands

Cells arising from *Villin* expressing progenitor cells are expressed in bright green, while DAPI is expressed in blue. Lineage tracing was performed from p1-p14. (Parappilly et al., unpublished)

Taken together, our results indicated that Brunner's glands are more closely related to a gastric origin than an intestinal origin.

Discussion

Over the years, an immense amount of research has been completed on the function and development of many intestinal cells, but the Brunner's gland has been largely overlooked. Perhaps this is because the gland is more clinically irrelevant compared to other parts of the human body, as clinical significance depends on the frequency of pathological cases. However, tumors arising in the Brunner's gland (both benign and malignant), are known to arise in humans and can cause serious and potentially life-threatening health issues. While there has been a fair amount of research completed on the function of the Brunner's gland, little is known about the developmental origins. The aim of this study was to provide insight into the factors and stem cells that contribute to the development of the Brunner's gland.

The first major finding in this study is that Brunner's glands arise from *Lrig1* positive cells and evidence for this was provided through developmental lineage tracing of *Lrig1cre-YFP* mice. In *Lrig1* knockout mice, there is a large increase in the expression of ErbB signaling, leading to cellular proliferation and duodenal adenomas (Wang, 2015). This finding provides evidence that *Lrig1* is a tumor suppressor, by negatively regulating cellular proliferation.

The second major finding in this study is that Brunner's glands do not seem arise from *Villin* positive cells. Developmental lineage tracing was used in order to test for the presence of the protein, and while *Villin* is present in most other intestinal epithelial cells, and it is interesting that they are not present in Brunner's gland cells. This result sheds light on the developmental origin of the gland, and we hypothesize

from these results that Brunner's gland cells may be more closely related to a gastric origin as opposed to an intestinal origin.

The third major finding in this study is that *SPDEF* is important for proper Brunner's gland development. *SPDEF* null mice had largely deformed and underdeveloped glands, and exhibited a loss in the major cellular marker AQP5. This aquaporin channel is used for the exchange of ions and water, so it is likely that some level of function is lost in the Brunner's gland of *SPDEF* null mice. However, antibody staining of *Muc5AC, Muc6, Muc4, ErbB2,* and *TFF2,* showed similar expression patterns in wildtype and SPDEF null mice. Therefore, while *SPDEF* is important for proper development of the Brunner's gland, it is likely that not all functions are impeded in the absence of *SPDEF*.

Due to the similarity of Brunner's glands to goblet cells, in that they are both mucin glycoprotein secreting cells, we had originally hypothesized that many of the factors important in the development of goblet cells would be shared with Brunner's gland cells. Our results how shown this is partially true. *SPDEF* is important for goblet cell and Brunner's gland cell differentiation, however from previous research one would think that *Gfi1* and *Atoh1* would also be important for its development as they are mediators in the secretory lineage pathway of intestinal stem cells (Krausova, 2014). While this is true for Goblet cells, the Brunner's gland exhibited no significant functional or structural changes in both *Gfi1* and *Atoh1* knockout mice. This seems contradictory to previous studies that stated that *SPDEF* expression is dependent on *Atoh1* and *Gfi1*, and in the absence of *Atoh1* and *Gfi1*, *SPDEF* expression would decrease (Shroyer et al., 2005). Perhaps the *Atoh1* and *Gfi1* knockouts did not decrease

SPDEF expression enough for it to have a significant affect on the Brunner's gland. It is also possible that there may be some other unknown proteins that play a role in the intestinal lineage allocation that have not been discovered yet, as shown in figure 11.

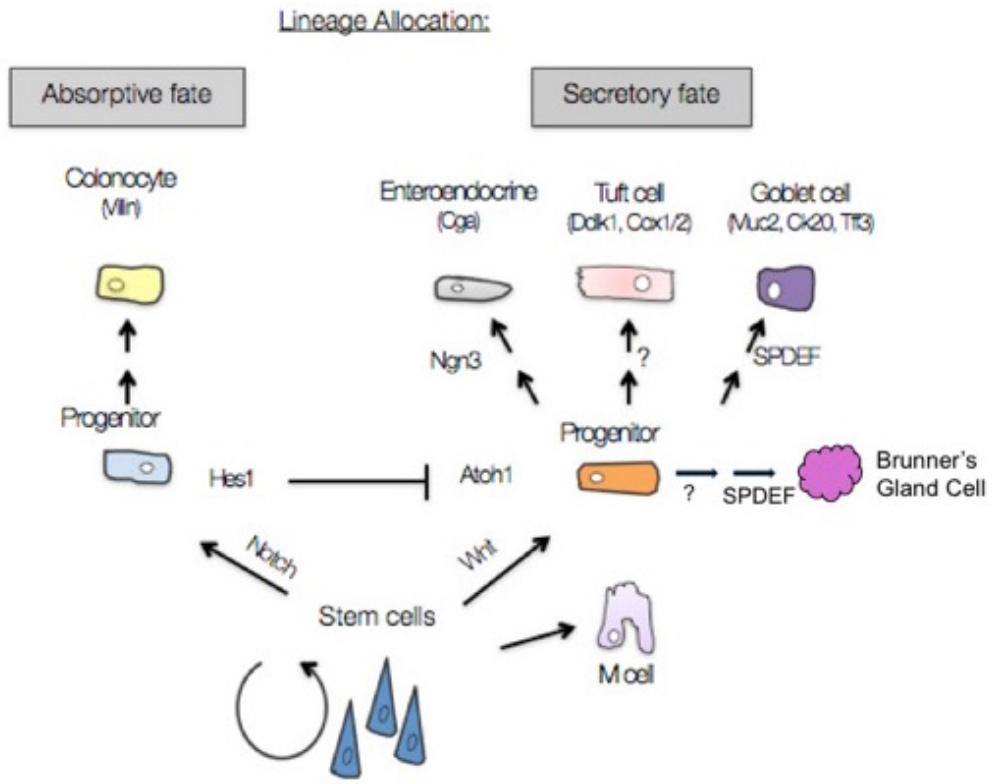

Figure 11: Updated Lineage Allocation for Intestinal Stem Cells

Our results show that *SPDEF* is important for Brunner's gland formation. However, it is unknown whether there are other proteins that contribute to its development. (Powell Lab – University of Oregon, Unpublished)

Using DBZ, *Notch* was inhibited in *SPDEF* null Brunner's glands. This was used as a control, as it forces all progenitor cells to take a secretory fate. In mutants without *NOTCH* inhibition, the underdevelopment of the Brunner's gland could be caused by progenitor cells taking an absorptive fate as opposed to a secretory fate. However, *SPDEF* knockouts remained largely underdeveloped and deformed in DBZ treated mice, and rules out the possibility that cells were only taking an absorptive fate

in the absence of *SPDEF*. This also tested for any contributions of *NOTCH* to Brunner's gland development, and from our results it seems that *NOTCH* does not have an important role in the gland's development.

There were some limitations with this study including that it only used qualitative observations for analysis and not quantitative observations. The use of quantitative observations such as cell counts, and areas of Brunner's glands would require that glands be oriented in the same direction, orientation, and plane. It is extremely difficult to account for these parameters, as paraffin embedding is outsourced to histology labs. There will also be natural variation between glands of different mice, and so these quantitative observations would only be practical if a large population of mice (>15) was used in order to normalize for this variation. Another limitation is that Brunner's glands could not be properly observed at p1 as they were largely undeveloped. The glands were extremely small, as most of their development occurs in the first 3 weeks after birth (Obuoforibo et al., 1977). This prevented proper analysis of gland at this stage, as they were too immature for qualitative observation.

In summary, our study provides insight into specific progenitor cells that contribute developmentally to the gland, and shed light on the molecular mechanisms important for proper formation. There is still much more to learn, as this study raises further questions on the interactions between the factors important for development, and invites the idea of completely new proteins that have not been discovered yet that may be in play. Regardless, this research will aid in future studies that focus on Brunner's gland development and homeostasis, and may be helpful in understanding how pathologies such as cancer arise in the gland.

Glossary

Adenoma: A benign tumor formed in epithelial tissues for glandular structures

Alcian Blue-PAS: A stain in histology that allows the differentiation of neutral mucins and acidic mucins

CreERT2 Recombinase: An enzyme derived from a bacteriophage that is used as genetic engineering tool to replace targeted sections of DNA upon activation of tamoxifen

Chyme: A mix of gastric secretions and partially digested food that passes from the stomach to the intestine

Developmental Lineage Tracing: The identification of a cell and all of its progeny cells through a shared marker during development

Differentiation: The process of a cell changing from one cell type to another cell type

Glycoprotein: A type of protein that has carbohydrate groups attached to its polypeptide chain

Helicobacter Pylori: A type of bacteria located in the stomach and intestine that is prone to causing ulcerations and cancer

Hematoxylin and Eosin: A basic and common stain used in histology that identifies the nucleus and cytoplasm of cells with different colored dyes

Histology: The study of tissues

Homeostasis: A stable equilibrium state of physiological processes in a cell

Husbandry: The breeding of animals

Knockout: Another word for “null,” and describes a genetic technique where an organism’s genes are made inactivated

Immunofluorescence: A method of visualizing protein expression using fluorescent antibodies that specifically bind to the protein of interest

Immunohistochemistry: A method of visualizing protein expression where the protein of interest is bound by specific antibodies

Lumen: The inside space of a tubular structure

Morphology: The study of the forms of cells

Mucin: A glycoprotein that is the main part of mucus

Null: Another word for "knockout," and describes a genetic technique where an organism's genes are made inactivated

Oligomerization: A chemical process that links single compounds in to a series of connected compounds

Progenitor Cell: A more highly differentiated stem cell

Proliferation: Rapid reproduction of cells

Serous cells: Epithelial cells that secrete fluid containing proteins, glycoproteins, or antibodies

Tamoxifen: A drug used to activate the CreERT2 recombinase

Transgenic: An organism that has genetic material artificially introduced from another unrelated organism

Transport (carrier) protein: A type of protein used in the movement of ions and molecules across a biological membrane

Ulceration: The process of developing a type of lesion that damages the skin or mucous membrane

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