Identification of novel gastric stem cell markers and investigation of Intestinal Stem Cell Cre-mediated Recombination

Theresa M. Keeley

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Identification of Novel Gastric Stem Cell Markers and Investigation of Intestinal Stem Cell Cre-mediated Recombination

by

Theresa M. Keeley

Thesis
Submitted to the Department of Biology
Eastern Michigan University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE
in
Molecular and Cellular Biology

Thesis Committee:

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November 2015
Ypsilanti, Michigan
Dedication

For my Grandpa, who first showed me the wonder of science and never stopped asking questions. I miss you.

Francis Guy Keeley
May 18, 1934–October 6, 2014
Acknowledgments

First, I would like to thank Dr. Linda Samuelson. Linda has been an amazing mentor who has truly been a great role model for me in science and in life.

I can’t say enough about the Samuelson Lab members, past and present. They are too numerous to name here, but I have had the tremendous luck to be surrounded by really smart, sweet people that turned into great friends through the years. Thank you for all you taught me and for all the great memories!

I would like to thank my committee members for taking the time to help me through this process.

Last, but not least, I would like to thank my fiancé, Derek Egeler. Thank you so much for all of the cleaning you did, all of the dinners you made, and all of the support and understanding you have given me!
Abstract

Very few genetic markers for gastric stem cells are currently described. Identifying new markers is important for increasing our basic understanding of gastric tissues and studying mechanisms of cancer development. Gastric and intestinal tissues share a common developmental program. Thus, intestinal stem cell genetic drivers were investigated for putative expression in gastric stem cells, utilizing Cre/Lox technology for lineage tracing. The recombination efficiencies of reporters with intestinal drivers and the effect of tamoxifen-induced tissue damage were also investigated.

It was discovered that higher doses of tamoxifen do not increase reporter activation in the intestine but induce gastric tissue damage. It was also determined that Bmi1 and Lrig1 are markers for stem cells throughout the glandular stomach. Thus, two new gastric stem cell drivers have been identified that will be useful for genetic manipulation of gastric epithelium.
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Introduction

Gastrointestinal (GI) tissues are highly dynamic, containing epithelia that undergo a rapid rate of renewal. Newly formed cells arise from highly active, multipotent, adult stem cells within the epithelia. The two hallmarks of an adult stem cell are 1) they can give rise to all differentiated cell types, and 2) they are capable of self-renewal. Dysregulation of stem cells is thought to be one of the crucial steps involved in the cellular transformation towards cancer development. Indeed, aberrations or mutations in adult GI stem cells can lead to hyperproliferation, resulting in tumorigenesis (57). Due to the potential for advancements in both the basic understanding of GI homeostasis as well as cancer cell development, identifying markers for GI stem cells is of the utmost importance to allow stem cell identification and genetic manipulation. In recent years, the GI stem cell field has advanced rapidly with the identification of several intestinal stem cell markers (4). The work presented herein will focus on investigating known intestinal stem cell markers, as well as identifying new molecular markers for gastric stem cells.

Adult Intestinal Stem Cells

The mouse intestinal epithelium is highly prolific, producing about 2.25 x 10^8 cells per day from approximately 7.5 x 10^5 crypts (12, 31), indicating the presence of highly active adult intestinal stem cells (ISCs). ISCs do not directly form differentiated cells; rather ISCs contribute to a progenitor pool of intermediate cells called transit-amplifying (TA) cells (Fig. 1). TA cells divide rapidly, approximately every 12–18 hours, up to six times before differentiating into one of the mature cell types (10).
Figure 1. Small Intestinal Epithelium. The image on the left is an H&E stained section of mouse duodenum. The image on the right (generated by A. Carulli, Samuelson Lab) is a schematic view of the small intestinal epithelium including the locations of the +4 and the Lgr5+ stem cells (arrows).
The specialized epithelial cells in the intestine form a monolayer that folds into invaginations called crypts and finger-like structures called villi, which protrude into the lumen (Fig. 1). The villi function to maximize tissue surface area for efficient nutrient absorption. The majority of differentiated epithelial cells in the small intestine are absorptive enterocytes. Secretory cells make up the other cell types, which include mucus-secreting goblet cells, hormone-secreting endocrine cells, tuft cells whose function is poorly described, and antimicrobial peptide-secreting Paneth cells (Fig. 1). After cells differentiate in the crypts, the majority will migrate up the villi over the course of a few days, before undergoing anoikis (programmed cell death) and being shed off the villus tips into the lumen. The Paneth cells are unique in that they migrate in the opposite direction, down to the base of the crypts where they lie intercalated between the ISCs, persisting for several weeks (Fig. 1).

Many signaling pathways are known to contribute to intestinal epithelial cell homeostasis. Of central importance are Wnt and Notch, which regulate stem cell proliferation and differentiation (10). Pathway dysregulation has been shown to lead to tumorigenesis. Wnt signaling, in particular, is upregulated in almost all cases of colon cancers, with gene mutations that constitutively activate Wnt signaling considered to be essential drivers of tumorigenesis in the colon (4). In addition, the Notch target gene Hes1 has been shown to be upregulated in colon adenocarcinomas (46). The cell (or cells) of origin of common GI cancers continues to be debated, although a stem cell source is currently favored.

**Lineage Tracing and Cre/Lox Technology**

Early studies utilizing long-term tritiated-thymidine incorporation into newly synthesized DNA identified putative ISCs located in an average of four cells from the base of the crypt,
referred to as the +4 cell (42) (Fig. 1). It had also been proposed that small cells in the base of the crypt, referred to as crypt base columnar (CBC) cells, were the stem cells of the intestine (6). This controversy as to the identity of the active ISC remained in the field for decades until a specific ISC marker, leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5), was identified by the Clevers lab in 2007 (5), utilizing lineage tracing, which is the current gold standard for testing stem cell activity. Lineage tracing uses a system of genetic modification to mark stem cells and their progeny. A common method for performing the genetic modification needed for lineage tracing utilizes Cre/Lox technology. In general, the Cre/Lox system involves the transgenic expression of the P1 bacteriophage-derived Cre recombinase enzyme (Cre) that targets a specific sequence known as LoxP for site-specific recombination (36). Transduction of this genetic system into mammalian cells allows the conditional manipulation of genes to study their function or to mark specific cell types.

An important advancement in Cre technology was the generation of inducible Cre drivers, which are Cre recombinase genes fused to a mutated estrogen receptor (CreERT) that can only be activated by the estrogen receptor antagonist tamoxifen (TX), not the endogenous receptor ligand, estradiol (58). For lineage tracing, CreERT activates the expression of a reporter gene, typically a fluorescent protein or other marker that can be visualized. The reporter is normally inserted into a ubiquitously expressed gene locus. The Rosa26 gene contains a ubiquitous promoter expressed throughout newborn and adult mouse tissues; therefore, transgenes inserted at this locus are expressed in all cells (14). Furthermore, Rosa26 does not have a necessary function; thus this gene can be genetically engineered without affecting viability or fertility. Many versions of Rosa26-reporter mice have been generated. These are comprised of the Rosa26 promoter followed by a transcriptional stop cassette flanked by LoxP sites (floxed).
upstream of a reporter gene (53). Activation of CreERT by TX allows the CreERT to enter the nucleus, where the recombinase will permanently excise the stop cassette between the LoxP sites and consequently allow the reporter gene to be expressed in that cell (Fig. 2A). As this is a permanent genetic change, the reporter will be expressed in the original CreERT-expressing cell, as well as in daughter cells. Therefore, if CreERT is expressed in a stem cell, a stripe of labeled cells is created along the intestinal crypt-villus axis or along the length of gastric glands (Fig. 2B,C) that will persist, reflecting the two properties of stem cells: self-renewal and generation of differentiated cell types.

**Intestinal Stem Cell Markers**

Recently, several molecular markers for ISCs have been identified, including *Lgr5* (5), which is a receptor for the Wnt agonist R-Spondin (9). *Lgr5* was initially identified through gene expression profiling as a Wnt target gene that was enriched in the proliferative crypt region of the small intestine. Based on those data, an Lgr5-eGFP-IRES-CreERT2 (Lgr5-CreERT2) reporter mouse strain was generated (5). With this mouse, Lgr5-expressing cells can be visualized with green fluorescent protein (GFP). Furthermore, the fused CreERT2 gene, when paired with a floxed allele, can inducibly knock out or activate genes specifically in Lgr5+ cells.

In order to test if *Lgr5* was indeed a marker for ISCs, lineage-tracing experiments were performed utilizing Lgr5-CreERT2 mice crossed to Rosa26-LoxP-Stop-LoxP-LacZ (Rosa-LacZ) reporter mice. Post-TX treatment, LacZ+ cells were visualized by X-gal staining for β-galactosidase (β-gal) (50). Utilizing this cross, lineage tracing experiments by Barker et al. (5) revealed that Lgr5+ cells were long-lived cells localized to the base of the crypts that can give rise to all intestinal epithelial cell types.
Figure 2. CreERT/Lox Activation. (A) Graphical depiction of a CreERT2 construct and Rosa26-LSL-reporter (Rosa-Tom) alleles before (left side) and after (right side) tamoxifen injection. The cell on the right side is red due to expression of the reporter. (B,C) Tomato expression in the stomach of Lgr5-CreERT2;Rosa-Tom mice one day (B) and seven days post-tamoxifen induction (C).
In addition, the GFP expression in these cells allowed for single cell sorting of Lgr5+ CBC stem cells by flow cytometry. When grown in culture, single Lgr5+ cells proliferated and formed crypt-like structures *in vitro*, termed organoids (48). Organoids represent a major advancement in the field by allowing *ex vivo* growth of physiologically relevant epithelium (2). Intact glands or crypts from stomach or intestine or single, isolated stem cells from either tissue can be plated into matrigel, which is a basement membrane mix extracted from Engelbreth-Holm-Swarm (EHS) sarcoma. Matrigel provides a three-dimensional space for the culture of long-lived, complex epithelial structures that can propagate indefinitely and can form differentiated cell types, suggesting stem cell activity (2). One example of the powerful translational opportunities that organoids represent was reported by Yui et al. (61) in a study that showed that single, marked Lgr5+ colonic stem cells could be cultured to form colonic organoids that were then introduced by enema into mice that had induced colonic damage. The transplanted colonic organoids integrated into the damaged colon and repaired it (61).

*Lgr5* is described as a marker for the CBC stem cell, which is thought to be the actively cycling stem cell in the intestine. However, markers for a distinct +4 ISC population have also been reported, including *Bmi1* (47), *mTERT* (32), *Lrig1* (43), and *Hopx* (56). Studies using these markers as CreERT drivers crossed with a Rosa reporter mouse strain showed that the +4 cells rarely form full lineage traces in the intestine (19–22). Interestingly, damage to the intestine resulting in CBC cell loss significantly increased lineage tracing events initiated from +4 ISC markers (32, 56, 60), indicating that the +4 cells may be quiescent stem cells (QSCs) that are not actively proliferating under homeostatic conditions, but can be activated under conditions of stress or injury to replace lost CBCs.
Adult Murine Gastric Epithelial Stem Cells

Gastric and intestinal tissues have many similarities, such as rapid epithelial cell renewal maintained by adult stem cells. Currently, very few markers for gastric stem cells (GSCs) have been identified (Table 1), and a paucity of such markers has made the study of cancer development challenging. Identifying new GSC markers is crucial to increase basic understanding of how epithelial cells in the stomach differentiate, as well as to aid in discovering steps and signaling pathways involved in the development of cancer.

The adult murine stomach is composed of three main parts: the squamous forestomach, and the glandular corpus and antrum (Fig. 3A). The glandular stomach epithelium is composed of cells that form tube-shaped structures called glands, the openings of which are in contact with the gastric lumen (Fig. 3B,D).

There are five main types of epithelial cells in the mouse corpus (Fig. 3C): the zymogenic chief cells found at the base of the glands, the acid-secreting parietal cells and mucous neck cells located in the neck region, the hormone secreting enteroendocrine cells—such as enterochromaffin-like (ECL) cells—scattered throughout the epithelium, and the surface mucous cells (or pit cells) located at the gland openings or pits (22). These specialized cells are constantly being replenished from stem and progenitor cells and have a varying yet predictable rate of turnover depending on the type of cell. The surface mucous cells, for example, turn over every 2–3 days (24), which is consistent with being in contact with the harsh acidic luminal environment, while the zymogenic chief cells, located at the base of the glands, have a longer lifespan of approximately 5 months (25). The proliferative zone, or isthmus, where new epithelial cells arise is located in the upper one-third of the gland (Fig. 3C, bracket). As a result, differentiating cells must migrate bi-directionally from the progenitors (23). Because the
<table>
<thead>
<tr>
<th>Marker</th>
<th>Gastric Tissue</th>
<th>Reference</th>
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<tr>
<td>Lgr5</td>
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<td>Barker et al., 2010</td>
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<tr>
<td>Sox2</td>
<td>Corpus and Antral Stem Cell</td>
<td>Arnold et al, 2011</td>
</tr>
<tr>
<td>*Villin-β-gal+</td>
<td>Antral Quiescent Stem Cell</td>
<td>Qiao et al., 2009</td>
</tr>
<tr>
<td>*Troy</td>
<td>Corpus Quiescent Stem Cell</td>
<td>Stange et al., 2013</td>
</tr>
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</table>

*Injury-related stem cell markers
Figure 3. Mouse Gastric Epithelium. (A) Gross view of an open mouse stomach with the three main parts outlined. (B) H&E stained section of the corpus. (C) Schematic representation of a corpus gland. (D) H&E stained section of the antrum. (E) Schematic representation of an antral gland and the positions of the Lgr5+ antral stem cells. For both C and E, the areas of the proliferating TA progenitors are highlighted with a bracket.
proliferating cells are present in the isthmus region of the glands, it is assumed that this is also the area that the stem cells reside. Electron microscopic (EM) studies in rats identified presumed stem cells in this area based on their simplistic appearance, which include features such as low cytoplasm to nucleus ratio, absence of many organelles and lack of granules (11). However, reliable molecular markers for these stem cells have yet to be identified.

The antrum has similar mucous cells to those of the corpus, though parietal and chief cells are absent in this region (Fig. 3E). There are also some distinct populations of endocrine cells, including G cells, which produce and secrete the hormone gastrin. Also, like the corpus, the antrum has a defined proliferative zone, but it is located just above the base of the glands (Fig. 3E, bracket).

Lineage tracing of Lgr5-CreERT2 crossed to Rosa26-LacZ mice revealed that the ISC marker Lgr5 is also an antral GSC marker, as Lgr5+ cells in the stomach can give rise to all epithelial cell types in the antrum (3). In addition, single Lgr5+ cells from the antrums of adult mice were isolated and when grown in culture, initiated organoids that contained all antral epithelial cell types, further illustrating their “stemness.” It was also discovered that Lgr5 has a varying temporal-spatial expression pattern. Lgr5+ cells are present throughout the newborn mouse glandular epithelium, but expression is subsequently restricted to the base of the antral glands as the stomach matures (3), eventually restricting expression in the corpus to the lesser curvature (38) (Fig. 3A). The rare corpus Lgr5+ cells co-label with chief cell markers and do not proliferate (38). Interestingly, if induced at birth, stripes of Lgr5-marked epithelial cells were present in the corpus as well (3). It is interesting to note that Lgr5-marked cells were still present in the corpus after eight months, well after all of the epithelial cells should have turned over. This may indicate a migration of some long-lived Lgr5-marked cells in the base of the immature
glands in young animals to the mid region of the corpus glands that persists in adults. Alternatively, the Lgr5-marked cells may give rise to adult GSCs in the corpus, which themselves do not express Lgr5.

In another recent paper, sex-determining region Y-box 2 (Sox2) was described as a putative stem cell marker for both the corpus and antrum (1). Utilizing a Sox2-CreERT2 crossed to a Rosa-YFP reporter mouse, they showed long-term labeling of cells in the glandular epithelium after TX induction. However, these cells appear to be rare, and one limitation of this study was that TX was administered at three weeks of age, prior to gastric gland maturation (26). Therefore, it is possible that the lineage tracing events in the corpus were a result of the activation of cells before the true separation of corpus and antral regions, akin to the Lgr5 corpus expression when induced at birth.

QSCs may be present in gastric tissue in addition to the intestine, as Qiao et al. (44) have described a putative QSC marker, villin, for the antral epithelium. Villin is an actin-binding protein that is expressed in all intestinal epithelial cells, including ISCs. However, β-gal expression driven by the villin promoter revealed rare Villin-β-gal+ cells in the antrum under homeostatic conditions, and when the stomach was subjected to inflammation injury by treatment with the cytokine interferon gamma (IÎn-γ), these Villin-β-gal+ cells actively proliferated and gave rise to all epithelial cell types (44).

More recently, Troy was identified as a marker for reserve stem cells in the corpus (55). By generating a Troy-eGFP-IRES-CreERT2 (Troy-eGFP) knockin mouse model, Stange et al. (55) were able to show that Troy-eGFP+ cells were localized to parietal and chief cells at the base of corpus glands. When crossed to a Rosa-LacZ reporter strain and induced with TX, rare, Troy-eGFP+ chief cells were able to form lineage ribbons. These tracing events increased
sixfold after damage to the active stem cells by an injection of fluorouracil (5-FU), which is a thymidylate synthase inhibitor that causes the death of rapidly dividing cells. Furthermore, single, isolated Troy-eGFP+ chief cells were able to form long-lived organoids in culture that could form differentiated mucous neck and pit cells (55).

During the normal course of lineage differentiation, mucous neck cells migrating down the gland toward the base activate the transcription factor Mist1 and further differentiate to become chief cells (18, 25). Moreover, previous evidence had suggested that chief cells could transdifferentiate into a metaplastic lineage called spasmolytic polypeptide-expressing metaplasia (SPEM), which arises from a loss of acid-secreting parietal cells (39) by re-expressing mucous neck cell markers such as Trefoil factor 2 (TFF2), also known as spasmolytic polypeptide (SP) (37). This transdifferentiation into SPEM was shown to arise from chief cells that did not express Lgr5 (38). Taken together, the data indicate that there may be some plasticity in stem cell differentiation and that chief cells may be able to de-differentiate to become stem cells upon tissue injury and active stem cell loss.

**Thesis Overview**

The overall hypothesis tested in this thesis was, given the similar properties between gastric and intestinal stem cells, some ISC markers will also be expressed in GSCs. This has already been shown to be true in the case of Lgr5, which marks ISCs and antral GSCs (3, 5); however, reliable GSC markers for actively cycling stem cells of the corpus have yet to be identified. To test this hypothesis, Lrig1-CreERT2 (43) and Bmi1-CreER (47) mice were utilized along with three different ROSA-reporter strains. First, intestinal tissues from these crosses were examined to determine proper activation of reporters.
Surprisingly, the results obtained from my studies were strikingly different from those previously published. Both Lrig1-CreERT2 and Bmi1-CreER were expressed in cells throughout the epithelium, rather than cells restricted to the +4 position, indicating that these markers are expressed in more than QISCs. Thus, before testing my hypothesis, further experiments were performed to define the expression of these markers in the intestine. To try to reconcile the observed differences in expression from those of published studies, four different published CreERT strains were each crossed to three different ROSA-reporter strains. In addition, varying doses of the activator TX was tested. Across all CreERTs, it was determined that different reporter strains had different amounts of activation; however, the dose of TX had no effect on the amount of reporter activation.

While increasing the dose of TX did not increase reporter activation in the intestine, TX has been shown to be injurious to gastric tissue at higher doses (19). Thus, gastric tissues were examined for damage caused by TX with the varying doses examined in the intestine. It was determined that TX at doses lower than those reported did indeed cause damage to parietal cells in the corpus.

Finally, to test my overarching hypothesis, the Lrig1-CreERT2 and Bmi1-CreER mice crossed to ROSA-Tom reporters (30) were examined and found to have reporter expression in gastric tissue after induction with TX. Over time, ribbons of labeled cells were formed in both corpus and antrum, which, through co-labeling with differentiated cell markers, were shown to represent all of the differentiated cell types. These labeled ribbons of cells persisted for more than one year, indicating marker activation in long-lived cells. Taken together, the data indicate that both Lrig1 and Bmi1 mark long-lived stem cells in both the corpus and antrum.
The thesis work presented here determines the activation of CreERT/reporter combinations with varying TX doses in the intestine, describes the potential gastric damage caused by TX administration, as well as identifies two new markers for corpus and antral epithelial cells in the stomach. Very little has been published concerning markers for GSCs and gastric QSCs (see Table 1). Discovery of new markers will help increase basic knowledge of how epithelial cells in the stomach differentiate and will aid in discovering steps involved in the development of gastric tumorigenesis.
Materials and Methods

Mice

Mice were obtained from Jackson Laboratories with the exception of VT2-CreERT2 mice (gift from S. Robine, Institut Curie) (Table 2). They were housed in ventilated and automated-watering cages under specific pathogen-free conditions. Mice (8 wks to 1 year old) were fasted overnight with free access to water prior to tissue collection. Male and female mice were studied. Mouse use was approved by the University of Michigan Committee on Use and Care of Animals (UCUCA).

Quantitative RT-PCR Measurement of mRNA Abundance

RNA Isolation

Total RNA was isolated from corpus, antrum, and proximal intestine using Trizol (Invitrogen) extraction. RNA was then purified and treated with DNase1 using RNeasy (Qiagen) columns per the manufacturer’s instructions.

Reverse Transcription and QPCR Reactions

Reverse-transcriptase (RT) reactions were performed with an iScript kit (Bio-Rad) according to the manufacturer’s instructions. Triplicates from each cDNA sample were amplified by quantitative PCR (QPCR) to measure specific mRNA concentrations. qRT-PCR was performed using an ICycler (Bio-Rad) with SYBR Green dye (Molecular Probes). Each 20-µl reaction contained 2 µl of reverse-transcribed product, PCR buffer (Invitrogen), 5.5 mM MgCl₂, 100 nM of each primer, SYBR Green, 10 nM fluorescein, 200 µM dNTPs, and 0.025 U Platinum Taq polymerase (Invitrogen). The following amplification conditions were used: 3 min at 95°C, 35
### Table 2: Mouse Strains

<table>
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<tr>
<th>Strain</th>
<th>Expression</th>
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<td>018418</td>
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<td>Rosa26-YFP</td>
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<td>006148</td>
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cycles of 9 s at 95°C, and 1 min at 60°C or 65°C, followed by 1 min at 55°C. Melt curve analysis was used to assess product purity. Expression levels were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GapDH), which remained the same in all samples.

**Primer Design and Validation**

Beacon software (BioRad) was used to design primer sequences. All quantitative RT-PCR (qRT-PCR) assays were validated by PCR of a dilution series to confirm appropriate quantitative amplification, and reaction specificity was confirmed by sequencing the PCR product. Primer sequences were as follows: H/K ATPase α Forward: 5’-TGT ACA CAT GAG AGT CCC CTT G-3’ and Reverse: 5’-GAG TCT TCT CGT TTT CCA CAC C-3’. GapDH Forward: 5’-TCA AGA AGG TGG TGA AGC AGG-3’ and Reverse: 5’-TAT TAT GGG GGT CTG GGA TGG-3’.

**TX Administration**

Tamoxifen (TX, Sigma) was dissolved in 100% EtOH (Fisher) (5 or 10% final EtOH concentration) and then added to corn oil (Sigma) for a final TX concentration of 10mg/ml or 20mg/ml, or TX was added directly to corn oil without EtOH to final concentrations of either 10mg/ml or 20mg/ml. TX was administered via either intraperitoneal (i.p.) injection or oral gavage. Corn oil with EtOH (5% final concentration) was injected as vehicle control.

**Histological Analysis**

**Tissue Preparation**

For paraffin sections, stomachs were removed and one half was fixed in 4% paraformaldehyde.
overnight. Paraffin sections (4 µm) were stained for hematoxylin and eosin (H&E) for evaluation of general histology.

For cryosections, stomachs were removed and one half was fixed in 4% paraformaldehyde for 1 hour then placed in 30% sucrose overnight at 4°C. Cryo blocks were made by embedding tissue in O.C.T. (Tissue Tek) and freezing on dry ice.

**Proliferation Analysis**

Mice were injected with the thymidine analog 5-Ethynyl-2’-deoxyuridine (EdU) (Invitrogen) (25 mg/kg) 1.5 hours before tissue collection. Paraffin sections (4 µm) were deparaffinized in xylenes, rehydrated in decreasing concentrations of EtOH from 100%, 90%, to 75%, and then PBS, then the EdU Click-it (Invitrogen) reaction performed according to the manufacturer’s instructions.

**LacZ Staining**

Cryosections (4 µm) were fixed in 4% paraformaldehyde for 5 min, rinsed 3 times (15 min) in X-gal wash buffer (0.1 M sodium phosphate pH 7.3 containing 2 mM MgCl₂ and 0.02% NP-40) and placed overnight in X-gal (5-bromo-4-chloro-3-indyl-β-D-galactosidase; Roche) staining solution (1mg/mL X-gal in N,N dimethylformamide, 5mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆·3H₂O in X-gal wash buffer) at 37°C, protected from light. Slides were then washed in X-gal buffer and counterstained with Neutral Red solution (5mg/mL neutral red (Sigma-Aldrich) in 1% glacial acetic acid), dehydrated, and coverslipped with Permount (Fisher).
**Immunostaining**

For H/K ATPase α staining, paraffin sections (4 µm) were deparaffinized in xylenes, rehydrated in decreasing concentrations of EtOH from 100%, 90%, to 75%, and then PBS, immunostained with H/K ATPase α (mouse, MBL) (Table 3) overnight at 4°C, then incubated with goat-anti-mouse Alexa 488 (Invitrogen) secondary and mounted with Prolong Gold plus 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Life Technologies) for nuclear staining. For co-staining, paraffin sections (4 µm) were deparaffinized in xylenes, rehydrated in decreasing concentrations of EtOH from 100%, 90%, to 75%, then PBS, and immunostained with red fluorescent protein (RFP) antibody (rabbit, Rockland) overnight at 4°C, and then differentiated cell markers (Table 3) overnight at 4°C, followed by incubation with appropriate secondary antibodies (Table 3) diluted 1:400, and mounted with Prolong Gold with DAPI. All primary and secondary antibodies were diluted in buffer containing 20% donkey serum and 1% bovine serum albumin (BSA) in 0.1% triton-PBS (TPBS). Antibody dilutions and information are in Table 3.

**Imaging**

Images were captured on a Nikon E800 microscope with Olympus DP controller software.

**Morphometric Analysis**

Image J (version 1.48v, Wayne Rasband, National Institutes of Health (http://imagej.nih.gov/ij)) was used to calculate epithelial length 5–9 field views per animal (n = 3 animals per age and genotype). The number of EdU-positive cells was counted, and the data were expressed as number of positive cells per length of epithelium in µm.
Table 3: Antibody Information

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*Secondary antibody from Invitrogen  
**Secondary antibody from Jackson ImmunoResearch
Statistics

GraphPad Prism software was used for statistical analysis and preparation of graphs.

Quantitative data are presented as means ± SE and analyzed by Student's t-test or 1-way ANOVA with Dunnett’s post-test. $P < 0.05$ was considered significant.
Results

Intestinal CreERT Activation

Disparate Intestinal CreERT-Driver Reporter Activation

The overall goal of my study was to identify reliable markers for gastric stem cells. Given the similarity between intestinal and gastric tissues and the identification of a shared intestinal and antral stem cell marker, Lgr5 (3, 5), two available quiescent intestinal stem cell (QISC) CreERT drivers—Bmi1-CreER (47) and Lrig1-CreERT2 (43)—were examined.

Bmi1-CreER and Lrig1-CreERT2 mice were crossed to Rosa26-LSL-tdTomato (Rosa-Tom) (30) mice and injected with TX at two months of age to induce recombination and subsequent Tomato reporter expression. Instead of the expected expression pattern in rare cells in the crypts as previously reported (43, 47) (Fig. 4A,C), multiple Tomato positive cells per crypt were seen 24 hours post-TX treatment (Fig. 4B,D). A similar pattern was observed with both CreERT drivers. In addition, both the TX-treated Bmi1-CreER;Rosa-Tom and Lrig1-CreERT2;Rosa-Tom intestine had Tomato-positive cells on the villi, which was surprising considering that both markers were reported as +4 QSC markers and were expected to only mark single cells in the crypts.

Unlike previously published reports, my study used a different Rosa reporter strain as well as disparate doses of TX. While my study used a Rosa-Tom reporter and a TX dose of 100mg/kg body weight, the previous studies used Rosa-LacZ reporter mice and 100–250mg/kg TX dose. In light of these differing results, it was decided that before the possible role of these genes as markers for gastric stem cells could be tested, the reporter activation of different intestinal CreERT drivers and specific Rosa reporters needed to be characterized.
Figure 4. CreERT Driver Differences in Rosa Reporter Expression. (A) X-gal staining of Bmi1-CreER;LacZ crypt 24 hours after TX induction (adapted from Sangiori and Capecchi, 2008) showing single labeled cells in the +4 position within the crypt. (B) tdTomato expression in Bmi1-CreER;Tom duodenum 24 hours after TX induction. (C) X-gal staining of Lrig1-CreERT2;LacZ duodenum 24 hours after TX induction (adapted from Powell et al., 2012). (D) tdTomato expression in the duodenum of Lrig-CreERT2;Rosa-Tom mice 24 hours after induction. (B,D) Data generated in the Samuelson Lab. tdTomato is red, DAPI for nuclear staining is in blue. (Note: images not to scale)
Toward this end, four intestinal CreERT drivers—Villin-CreERT2 (13), Lgr5-CreERT2 (5), Bmi1-CreER (47), and Lrig1-CreERT2 (43)—were each crossed to three different ROSA26 reporters—LacZ (53), tdTomato (Tom) (30), or yellow fluorescent protein (YFP) (54) (Table 3). Additionally, varying doses of TX were tested to determine if TX concentration affected reporter activation. Therefore, for each CreERT;Rosa-reporter cross, four treatments were administered; vehicle, 50mg/kg (low), 100mg/kg (medium) or 200mg/kg (high) of TX, and tissues were collected 24 hours later to assess the baseline expression pattern for each CreERT driver.

*Villin-CreERT2 Intestinal Activation*

The Villin-CreERT2 (VT2) crosses were studied first to establish baseline reporter activation with ubiquitous CreERT expression, as Villin is expressed throughout the intestinal epithelium in differentiated, stem, and progenitor cells. Upon examination of reporter expression among all TX doses studied, as expected, all epithelial cells had expressed tdTomato (Fig. 5A–C). However, only approximately 75% of the epithelial cells expressed the Rosa-LacZ reporter (Fig. 5D–F). Morphometric counting of the percentage of crypts that had reporter activation revealed no difference in the amount of activation between the three TX doses with each reporter (Fig. 5G). However, comparisons of the same dose between the two reporters showed significantly greater crypt activation with the Rosa-Tom reporter compared with the Rosa-LacZ reporter (Fig. 5H). Since activation frequency was similar among the TX doses tested for each cross, samples were pooled for each reporter to reveal that 79 ± 2% crypt activation occurred with the Rosa-LacZ reporter, while 100% crypt activation was observed with the Rosa-Tom reporter (Fig. 5I).
Figure 5. Villin-CreERT2 Rosa26 Reporter Expression. Cryosections of Villin-CreERT2;Tom (A–C), or Villin-CreERT2;LacZ (D–F) 24 hours after low (A,D), medium (B,E), or high (C,F) dose TX administration. (A–C) tdTomato (red) with DAPI (blue) nuclear stain. (D–F) LacZ (blue) with neutral red counterstain. (G–I) Morphometric counting of the proportion of crypts activated for reporter expression with each TX dose. (G) Comparison of crypt activation between doses for each reporter. (H) Comparison of crypt activation with the same dose between the two reporters. (I) Comparison of pooled LacZ and Tom samples to get total percentages for all doses. N=3–4 mice/treatment. n.s.=not significant, Comparisons are between LacZ *p=0.0318, ***p=0.0006, ****p<0.0001 vs. Tom for each dose by Student’s t-test. Scale=100μm.
**Lgr5-CreERT2 Intestinal Activation**

Next, Lgr5-CreERT2 crosses were examined. *Lgr5* is a known intestinal stem cell marker, with expression in the CBC stem cells at the base of the crypts (5) (See Fig. 1). With both Rosa-LacZ and Rosa-Tom reporter crosses, some, but not all of the crypts had activated reporter expression in cells at the base of the crypts (Fig. 6A–F). Although Lgr5+ stem cells are uniformly distributed in the base of all crypts, the Lgr5-CreERT2 mouse model exhibits mosaicism, resulting in patchy activation that was originally reported (5). Morphometric counting of the crypts expressing the tdTomato or LacZ reporter revealed that there was no difference in the amount of activation between TX doses (Fig. 6G), similar to results observed with the Villin-CreERT2 studies. It was determined, however, that there was a significant difference in the amount of crypt activation between the two reporters (Fig. 6H). Pooling the samples for each reporter showed 21 ± 2% activation with the Rosa-LacZ reporter, and 43 ± 3% with the Rosa-Tom reporter (Fig. 6I).

**Bmi1-CreER Intestinal Activation**

We next utilized the Bmi1-CreER driver to examine reporter activation in +4 QSCs, using both the Rosa-Tom and Rosa-LacZ reporters and differing doses of TX as used above (Fig. 7). In stark contrast to previous studies, which reported single-labeled cells at the base of the crypts (see Fig. 4A), my studies showed that epithelial cells on the villi were also labeled 24 hours after TX treatment (Fig. 7 arrows). The Rosa-LacZ reporter was commonly activated in numerous cells within the crypts 24 hours post-TX with all doses, as well as in occasional single cells on the villi (Fig. 7D–F). Similarly, visualization of the tdTomato reporter at the same time post-activation revealed multiple labeled cells per crypt, as well as frequent single cells along the
Figure 6. Lgr5-CreERT2 Rosa26 Reporter Expression. Cryosections of Lgr5-CreERT2;Tom (A–C), or Lgr5-CreERT2;LacZ (D–F) 24 hours after low (A,D), medium (B,E), or high (C,F) dose TX administration. (A–C) tdTomato (red) fluorescence with DAPI (blue) nuclear stain, (D–F) LacZ stained for β-gal (blue) with neutral red counterstain. (G–I) Morphometric counting of the % of crypts activated for each TX dose. (G) Comparison of the % of crypts activated between doses for each reporter. (H) Comparison of the % of crypts activated with the same dose between the two reporters. (I) Comparison of pooled LacZ and Tom samples to get total percentages for all doses combined. N=4–6 mice/treatment. n.s.=not significant. Comparisons are between LacZ *p<0.05, **p=0.0016, ****p<0.0001 vs. Tom for each dose by Student’s t-test. Scale=100μm.
Figure 7. Bmi1-CreER Rosa26 Reporter Expression. Cryosections of Bmi1-CreER;Tom (A–C), or Bmi1-CreER;LacZ (D–F) 24 hours after low (A,D), medium (B,E), or high (C,F) dose TX administration. Arrows indicate cells labeled on the villi. (A–C) Rosa-tomato (red) with DAPI (blue) nuclear stain. (D–F) Rosa-LacZ (blue) with neutral red counterstain. (G–I) Morphometric counting of the proportion of crypts activated for reporter expression with each TX dose. (G) Comparison of crypt activation between doses for each reporter. (H) Comparison of crypt activation with the same dose between the two reporters. (I) Comparison of pooled LacZ and Tom samples to get total percentages for all doses. N=3–4 mice/treatment. n.s.=not significant. Comparisons are between LacZ **p<0.01, ***p=0.0007, ****p <0.0001 vs. Tom for each dose by Student’s t-test. Scale=100µm.
villi (Fig. 7A–C). As before, no difference was observed between doses with either reporter (Fig. 7G), but a significant difference in the amount of activation with the Rosa-Tom reporter compared to the Rosa-LacZ reporter was observed (Fig. 7H). Combining the samples for each reporter showed 14 ± 2% crypt activation with the Rosa-LacZ reporter, compared to the significantly higher 68 ± 5% activation with the Rosa-Tom reporter (Fig. 7I).

*Lrig1-CreERT2 Intestinal Activation*

Analysis of Lrig1-CreERT2 also showed different results from those previously published (43). Crossing the Rosa-LacZ reporter to the Lrig1-CreERT2 driver revealed fully labeled crypts, as well as cells extending up the villi (Fig. 8D–F). Likewise, with the Rosa-Tom reporter, entire crypts were labeled along with cells on the villi (Fig. 8A–C). No difference in the amount of activation was observed between TX doses with either reporter (Fig. 8G); however, the average Rosa-LacZ crypt activation of 75 ± 3% was significantly lower than the 100% activation observed with the Rosa-Tom reporter (Fig. 8I).

*Rosa26-LSL-YFP*

A third ROSA26 reporter, YFP (54), was also tested with the VT2-CreERT2, Bmi1-CreER, and the Lrig1-CreERT2 drivers with a medium dose of TX, and tissue was collected 24 hours post-treatment. Cryosections were immunostained with an anti-GFP antibody to detect the YFP reporter, since YFP is a spectral variant of GFP. In contrast to the Rosa-LacZ or Rosa-Tom reporters, a 24-hour chase period was insufficient to allow detection of the Rosa-YFP reporter with any of the three CreERT drivers (Fig. 9A and data not shown). Further investigation with a longer chase period revealed that the YFP signal could be detected 36 hours and longer post-
Figure 8. Lrig1-CreERT2 Rosa26 Reporter Expression. Cryosections of Lrig1-CreERT2;Tom (A–C), or Lrig1-CreERT2;LacZ (D–F) 24 hours after low (A,D), medium (B,E), or high (C,F) dose TX administration. (A–C) tdTomato (red) with DAPI (blue) nuclear stain. (D–F) LacZ (blue) with neutral red counterstain. (G–I) Morphometric counting of the proportion of crypts activated for reporter expression with each TX dose. (G) Comparison of crypt activation between doses for each reporter. (H) Comparison of crypt activation with the same dose between the two reporters. (I) Comparison of pooled LacZ and Tom samples to get total percentages for all doses. N=3 mice/treatment. n.s.=not significant. Comparisons are between LacZ *p<0.05, ***p=0.0003, ****p<0.0001 vs. Tom for each dose by Student’s t-test. Scale=100µm.
Figure 9. Rosa26-YFP Reporter Expression. Cryosections of Lrig1-CreERT2;YFP 24 hours (A), 3 days (B), or 5 days (C) after TX administration. GFP immunostaining (green) with DAPI (blue) nuclear stain. Scale=100µm.
induction with TX (Fig. 9B,C). Lgr5-CreERT2 could not be examined with the Rosa-YFP reporter 24 hours post-induction because the GFP antibody also recognizes the GFP present in the Lgr5-CreERT2 construct.

Spontaneous CreERT Activation

Vehicle-treated controls were also examined to determine if spontaneous activation of the reporter could be detected. Low levels of reporter activation were detected with all four CreERT drivers (Fig. 10), indicating that a small amount of CreERT protein-induced recombination occurred in the absence of TX. Morphometric counting of the incidences of activation revealed low activation with all CreERT drivers and both Rosa-LacZ and Rosa-Tom reporters (Fig. 10I). Interestingly, in most of these cases with the tdTomato reporter, ribbons of labeled cells were detected, indicating spontaneous activation in stem cells that enabled persistence of the reporter signal (Fig. 10A–H). With the LacZ reporter, spontaneous activation was also observed with all CreERTs (Fig. 10E–H); however, ribbons of labeled cells were only observed with the VT2- and Lrig1-CreERT2s (Fig. 10E,H). Spontaneous reporter activation was only observed when both a CreERT driver and reporter allele was present; no reporter activation was detected in mice lacking CreERT drivers (data not shown).

Identification of Gastric Stem Cells

Intestinal Stem Cell Marker Genes Are Transcribed in the Stomach

In order to test the possible role of intestinal stem cell CreERT drivers as markers for gastric stem cells, I first investigated whether intestinal stem cell marker genes were also transcribed in gastric tissue. Toward this end, stomachs were harvested from wild-type mice and
Figure 10. Spontaneous Rosa26 Reporter Expression. Cryosections from VT2-CreERT2 (A,E), Lgr5-CreERT2 (B,F), Bmi1-CreER (C,G), and Lrig1-CreERT2 (D&H) were either imaged for tdTomato (red) with DAPI (blue) nuclear stain (A–D), or stained for β-gal (blue) with neutral red counterstain (E–H). Lgr5-CreERT2 and Bmi1-CreER with the Rosa-LacZ had activated crypts (F&G Arrowheads), but no lineages traces observed. Morphometric counting of the proportion of crypts activated without TX treatment for each Cre driver and reporter (I). N=1–4 mice/treatment. Scale=100µm.
separated into corpus and antrum. Duodenum (proximal intestine) was also collected as a positive control. In support of my hypothesis, it was found by qRT-PCR that Bmi1 and Lrig1 were expressed in both gastric corpus and antrum (Fig. 11). Moreover, Bmi1 mRNA abundance was 3.5-fold higher in the corpus and 15-fold higher in the antrum compared to the intestine (Fig. 11A). Lrig1 mRNA abundance was about 13-fold higher in the corpus compared to intestine, and approximately 5.5-fold higher in the antrum than the intestine (Fig. 11B).

*Bmi1-CreER and Lrig1-CreERT2 Are Active in the Corpus and Antrum*

Having determined that Bmi1 and Lrig1 were indeed expressed in gastric tissues, Bmi1-CreER and Lrig1-CreERT2 were crossed to Rosa-Tomato reporter mice (Bmi1-CreER; Tom and Lrig1-CreERT2; Tom), as that had been determined to be the most efficient reporter of those tested (see Figs 7,8). The medium dose of TX (100mg/kg) was injected into adult mice and gastric tissue was collected 24 hours post-treatment to determine which cells expressed Bmi1-CreER and Lrig1-CreERT2 in the stomach. Both Bmi1-CreER; Tom and Lrig1-CreERT2; Tom had activated tdTomato expression in single, scattered cells in the epithelium of the corpus (Fig. 12A,C) and antrum (Fig. 12B,D).

*Bmi1 and Lrig1 Mark Stem Cells in the Corpus and Antrum*

To determine if Bmi1-CreER and Lrig1-CreERT2 drivers were active in gastric stem cells, tissues were examined for long-lived lineage traces. Bmi1-CreER; Tom and Lrig1-CreERT2; Tom mice were injected with the medium TX dose and collected 1 year later. Complete gland labeling for both Bmi1-CreER and Lrig1-CreERT2 in the corpus (Fig. 13A,C) and antrum (Fig. 13B,D) was observed. The presence of fully labeled glands that persist after 12
Figure 11. Intestinal Stem Cell Markers Are Expressed in Gastric Tissues. (A&B) qRT-PCR for *Bmi1* (A) or *Lrig1* (B) gene expression in corpus, antrum and intestine. N = 3 mice for each tissue.
Figure 12. ISC CreERT Drivers Are Expressed in Gastric Tissues. tdTomato (red) expression in Bmi1-CreER;Tom corpus (A) and antrum (B) and Lrig1-CreERT2;Tom corpus (C) and antrum (D) 24 hours after medium dose TX (100mg/kg) administration. DAPI for nuclear staining in blue. Scale = 100μm.
Figure 13. *Bmi1* and *Lrig1* Are Expressed in Gastric Stem Cells. Bmi1-CreER;Tom corpus (A) and antrum (B) and Lrig1-CreERT2;Tom corpus (C) and antrum (D) 12–13 months after a single medium TX dose (100mg/kg). tdTomato (red) with DAPI for nuclear staining (blue). Scale = 100μm.
months in the corpus and antrum, well after the time necessary for turnover of all differentiated epithelial lineages, indicates that both Bmi1 and Lrig1 mark long-lived stem cells in the stomach.

To confirm that Lrig1+ stem cells give rise to all differentiated cell types in both corpus and antral epithelium, co-immunostaining was performed to identify cell lineage markers (Table 4) that also expressed the Rosa-Tom lineage trace in Lrig1-CreERT;Tom gastric tissue 13 months after TX induction (Fig. 14). Co-labeling was confirmed for all differentiated cell types in the corpus, including parietal cells (Fig. 14A), chief cells (Fig. 14B), endocrine cells (Fig. 14C), mucous neck cells (Fig. 14D), and surface mucous cells (Fig. 14E). Co-labeling was also confirmed in the antral cell types, including endocrine cells (Fig. 14F), deep mucous cells (Fig. 14G), and surface mucous cells (Fig. 14H). Parietal and chief cells are not present in the mature antrum (Fig. 3).

Co-immunostaining was also performed to test if Bmi1+ stem cells gave rise to all differentiated cell types in the gastric epithelium (Fig. 15). Twelve months after TX administration, Rosa-Tom reporter expression was indeed confirmed in parietal cells (Fig. 15A), chief cells (Fig. 15B), endocrine cells (Fig. 15C), mucous neck cells (Fig. 15D), and surface mucous cells (Fig. 15E) in the corpus. In the antrum, endocrine cells (Fig. 15F), deep mucous cells (Fig. 15G), and surface mucous cells (Fig. 15H) co-labeled with the tdTomato reporter.

Taken together, these data provide evidence that Bmi1 and Lrig1 are bona fide stem cell markers in the epithelium of both the corpus and antrum.
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Figure 14. *Lrig1* Gives Rise to All Lineages in the Gastric Epithelium. *Lrig1*-CreERT2;Tom cryosections from samples collected 13 months post-TX were immunostained for differentiated cell markers to identify co-labeled cells with tdTomato. Parietal cells (A), chief cells (B), endocrine cells (C, arrows), mucous neck cells (D), surface mucous cells (E) in the corpus, and endocrine cells (F, arrows), deep mucous cells (G), and surface mucous cells (H) in the antrum are in green. Red is tdTomato or RFP antibody with DAPI for nuclear staining in blue. Scale = 50μm.
Figure 15. *Bmi1* gives Rise to All Lineages in the Gastric Epithelium. Bmi1CreER;Tom cryosections from samples collected 12 months post-TX were immunostained for differentiated cell markers to identify co-labeled cells with tdTomato. Parietal cells (A), chief cells (B, arrows), endocrine cells (C, arrows), mucous neck cells (D), surface mucous cells (E) in the corpus, and endocrine cells (F, arrows), surface mucous cells (G), and deep mucous cells (H) in the antrum are in green. Red is tdTomato or RFP antibody with DAPI for nuclear staining in blue. Scale = 50µm.
Tamoxifen Damage in Gastric Epithelium

Gastric Epithelial Damage in Response to Tamoxifen

TX has been shown to cause damage to mouse gastric tissue at doses of 150mg/kg body weight and higher (19). While investigating the CreERT;Rosa-Reporter crosses for reporter activation with different TX doses in the small intestine, gastric tissue was also collected to examine potential damage to the epithelium caused by TX administration. Gastric paraffin sections 24 hours after vehicle, low, medium, or high TX-treatment (3–5 mice/treatment) were H&E-stained to examine general morphology of the epithelium (Fig. 16A–D). As predicted, the high TX dose caused substantial cellular damage in the corpus, with gland atrophy and delaminated cells frequently observed (Fig. 16D). Examination of parietal cells by immunostaining for the parietal cell marker H/K ATPase revealed a marked decrease in the number of cells 24 hours after high TX doses (Fig. 16H). Parietal cell loss was confirmed by qRT-PCR analysis, with a significant decrease in \( H/K \text{ATPase a (Atp4a)} \) mRNA expression (Fig. 16I). Decreased \( Atp4a \) was also observed with the medium TX dose (Fig. 16I) although this dose was lower than the previously reported minimal damaging dose (19). Gastric tissues were also examined three days post-TX treatment, as three days was previously found to be the height of the damage response (19). At this timepoint, the morphology by H&E (Fig. 16J–M) as well as H/K ATPase immunostaining (Fig. 16N–Q) revealed that the parietal cell damage progressed further in both the medium and high doses. mRNA expression of \( Atp4a \) continued to be significantly reduced with the medium and high TX doses, and interestingly, the low dose also showed a significant reduction in \( Atp4a \) expression (Fig. 16R).
Figure 16. TX Treatment Causes Parietal Cell Loss. Mice were treated with vehicle (A,E,J,N), low (50mg/kg) (B,F,K,O), medium (100mg/kg) (C,G,L,P), or high (200mg/kg) (D,H,M,Q) doses of tamoxifen and examined 24 hours (A–I) or 3 days (J–R) later. Paraffin sections were H&E stained to examine tissue morphology (A–D and J–M) and immunostained for H/K ATPase (green) to examine parietal cells with DAPI (blue) nuclear stain (E–H and N–Q). (I,R) qRT-PCR for Atp4a mRNA expression with the various doses of TX 24 hours (I) and 3 days (R) later. N=3–4 mice/treatment. *p<0.05, **p<0.01, ***p<0.001 vs vehicle by 1-way ANOVA. Scale=100μm.
**Tamoxifen Damage Activates Stem and Progenitor Cell Proliferation**

To examine if proliferation was affected by TX treatment, mice were injected with EdU 1.5 hours prior to tissue collection. Paraffin sections were stained for EdU (Fig. 17A–D) and morphometric analysis was performed to count the number of EdU+ cells with each treatment. In the corpus, it was determined that there was no difference between low and medium TX doses; however, there was a significant increase in the number of proliferating cells in the corpus with the high dose treatment (Fig. 17E). In contrast, by EdU staining (Fig. 17F–I) and morphometrics (Fig. 17J), the antrum showed no difference in proliferation among the three doses of TX. Three days post-TX, the increased proliferation effect in the corpus (Fig. 17K–N) expanded to include the medium dose of TX (Fig. 17O), further indicating that this dose does in fact cause damage to the gastric epithelium. The antrum at three days continued to have no changes in proliferation (Fig. 17P–T). Taken together with the parietal cell expression data from Fig. 16, these data indicate that all doses of TX examined induced a damage response in the gastric epithelium, although the low dose had a more mild effect as it did not activate progenitor cell proliferation.

**Gastrin Is Not Required for the Proliferative Response to Tamoxifen Damage**

The growth hormone gastrin has been shown to be instrumental for increased proliferation in the corpus following parietal cell loss (20). To determine if gastrin is responsible for the increased proliferation observed after high doses of TX, gastrin-knockout (GKO) mice (15) were treated with either vehicle or a high dose of TX. Paraffin sections were H&E-stained for general morphology, which showed disrupted glands and delaminated cells in the high dose treatment compared to vehicle, suggesting that a TX damage response similar to wild-type mice
Figure 17. TX Treatment Causes Increased Proliferation in the Corpus. Wild-type mice treated with vehicle (A,F,K,P), low (B,G,L,Q), medium (C,H,M,R), or high (D,I,N,S) doses of TX either 24 hours (A–J) or 3 days (K–T) before tissue collection. Paraffin sections were stained for EdU (green) with DAPI (blue) nuclear stain. (E,J) Morphometric counting of EdU+ cells in the corpus (E) and antrum (J) 24 hours after TX treatment. (O,T) Morphometric counting of EdU+ cells 3 days after TX treatment in the corpus (O) and antrum (T). N=3–4 mice/treatment. ***p<0.001, ****p<0.0001 vs. vehicle by 1-way ANOVA. Scale=100μm.
was observed in GKO mice (Fig. 18A,B). Accordingly, a loss of parietal cells was observed by immunostaining for H/K ATPase (Fig. 18C,D). Examination and quantification of EdU+ positive cells in the corpus revealed a significant increase in the number of proliferating cells with the high dose of TX in GKO mice (Fig. 18E–G).

Furthermore, examination of GKO mice three days post-TX treatment showed a similar response as the wild-type mice with the various doses of TX (Fig. 19A–D). The low dose had no effect, while the medium and high doses resulted in a significant increase in proliferation in the corpus (Fig. 19E–I). These data, together with the 24-hour post-TX data from Fig. 18, suggest that gastrin is not causative nor is it required for the resulting increase in proliferation after parietal cell loss due to TX.

*EtOH Does Not Cause Gastric Damage*

To test the possibility that the ethanol (EtOH) used to dissolve the TX into solution is a potential cause of the toxicity to parietal cells and gastric damage, TX was made without EtOH and injected at medium and high doses. At 24 hours post-TX without EtOH, mRNA expression of *Atp4a* was significantly reduced with both doses (Fig. 20A). In addition, proliferation in the corpus was significantly increased in both wild-type (Fig. 20B) and GKO (Fig. 20C) mice treated with TX without EtOH. Mice treated with TX without EtOH were also examined three days post-treatment, and it was determined that the increased proliferation observed at 24 hours persisted at three days post-TX without EtOH in both wild-type (Fig. 20D) and GKO (Fig. 20E) tissue. These data indicate that it is indeed the TX, and not EtOH, that causes the parietal cell damage.
Figure 18. Gastrin Is Dispensable for the TX Damage Response. Gastrin-deficient (GKO) mice were treated with vehicle (A,C,E) or high (B,D,F) doses of TX and examined 24 hours later. Paraffin sections were H&E stained to examine tissue morphology (A,B), immunostained for H/K ATPase to examine parietal cells (green, C,D), and stained for the proliferation marker EdU (green, E,F) with DAPI (blue) nuclear stain. (G) Morphometric counting of EdU+ cells in the corpus of GKO mice with each treatment. N=3 mice/treatment. ****p<0.0001. Scale=100μm (A,B,E,F), 50 μm (C,D).
Figure 19. Gastrin Is Not Required for Increased Proliferation after TX Damage. GKO mice were treated with vehicle (A,E), low (B,F), medium (C,G), or high (D,H) doses of TX and examined three days later. Paraffin sections were stained for H&E to examine tissue morphology (A–D) or with EdU (green) with DAPI (blue) nuclear stain to examine proliferation in the corpus (E–H). (I) Morphometric counting of EdU+ cells in the corpus of GKO mice three days post-TX. N=3–4 mice/treatment. ****p<0.0001 vs. vehicle by 1-way ANOVA. Scale=100μm
Figure 20. EtOH in TX Does Not Cause Gastric Damage. Wild-type (A,B,D) or GKO (C,E) mice were injected with vehicle, medium, or high doses of TX without EtOH and examined 24 hours (A–C) or three days (D,E) later. (A) qRT-PCR for \textit{Atp4a} mRNA expression with vehicle, medium or high doses of TX without EtOH 24 hours after treatment. (B–E) Morphometric counting of EdU+ cells in the corpus 24 hours (B,C) or three days (D,E) after treatment with TX without EtOH. N=3–4 mice/treatment. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. vehicle by 1-way ANOVA.
An additional test was performed to determine if the mode of TX administration affected gastric epithelial damage. Wild-type mice were given a high dose of TX by oral gavage, as well as TX without EtOH to determine if direct luminal contact by the EtOH caused gastric damage. Tissue morphology was examined 24 hours post-treatment by H&E (Fig. 21A–C). As observed with i.p. injections, the tissue appears damaged after oral gavage administration, with loss of parietal cells and the presence of delaminated cells in the epithelium. H/K ATPase immunostaining confirmed a loss of parietal cells with a high dose of TX (Fig. 21E) or TX without EtOH (Fig. 21F) by oral gavage, confirming that EtOH does not cause damage and demonstrating that TX damages the gastric epithelium regardless of the route of administration.
Figure 21. Oral Gavage With or Without EtOH Does Not Ameliorate TX Damage. Wild-type mice were treated with vehicle (A,D), high TX with EtOH (B,E), or high TX without EtOH (C,F). (A–C) H&E stained paraffin sections to examine morphology. (D–F) H/K ATPase (green) immunostained sections to examine parietal cells with DAPI (blue) for nuclear staining. Scale=100μm.
Discussion

Summary

The goal of this thesis project was to identify gastric stem cell genetic markers. A lineage tracing approach was used to determine if previously described intestinal stem cell specific, tamoxifen-regulated CreERT drivers would induce reporter lineage traces in the glandular stomach. Identifying new markers for gastric stem cells will allow for stem cell identification and genetic manipulation in genetically engineered mouse models.

ISC markers were tested for expression in gastric tissues, and both Bmi1 and Lrig1 mRNA were highly expressed in both corpus and antrum. Accordingly, Bmi1-CreER;Tom and Lrig1-CreERT2;Tom mice showed widespread reporter activation in the gastric epithelium 24 hours post TX-treatment. The presence of lineage tracing in gastric tissues of Bmi1-CreER;Tom and Lrig1-CreERT2;Tom mice one year after activation indicates that both of these CreERT drivers are present in corpus and antral stem cells, in addition to differentiated cells. These observations were confirmed by co-expression of the Rosa-Tom reporter and differentiated cell markers of all gastric epithelial cell lineages.

Importantly, when the intestines of Bmi1-CreER;Tom and Lrig1-CreERT2;Tom mice were examined after TX treatment, the reporter expression pattern did not agree with previously published data (43, 47). Instead of specific labeling of quiescent stem cells (QSCs), widespread labeling of differentiated cell populations was observed. To try to elucidate the potential cause of the differences in my results from the published findings, the possible effects of varying ROSA-reporters and TX doses were investigated. For this analysis, four intestinal CreERT drivers were crossed to three Rosa26-reporter strains. Together, the data show differences in the amount of reporter activation with different reporter strains. The Rosa-Tom reporter strain is the
most highly activated across all CreERT drivers tested when compared to Rosa-LacZ and Rosa-YFP. Surprisingly, varying the dose of TX had no effect on the number of intestinal crypts activated, regardless of the CreERT driver. In contrast to Rosa-Tom or Rosa-LacZ, the Rosa-YFP reporter was not detected 24 hours after activation. Furthermore, the Rosa-LacZ and Rosa-Tom reporters had small amounts of spontaneous reporter activation without TX administration. These findings suggest that different Rosa-reporter constructs have varying levels of sensitivity, yet within each construct with the doses tested here, different TX doses do not affect activation.

These studies also showed that even low doses of TX cause damage to the gastric epithelium (Table 5). While only the high dose appeared to damage the corpus 24 hours post-treatment, mRNA expression of the parietal cell marker Atp4a was significantly decreased with the medium dose as well. With all doses examined, this damage effect was most apparent three days post-TX, with Atp4a expression significantly decreased with all doses and increased proliferation observed with the medium and high doses. In other mouse models, the growth hormone gastrin has been shown to be required for a resulting increase in the number of proliferating cells after parietal cell loss (20, 39). Surprisingly, the data presented here indicate that gastrin is not required for the proliferation increase after parietal cell death caused by TX damage. In addition, the presence of EtOH in the TX solution does not cause the resulting damage effect nor does the route of administration.

**Gastric Stem Cell Marker Identification**

Validation of stem cell markers makes use of Cre/Lox technology to activate reporters to visualize long-term lineage tracing (see Fig. 2). New GSC markers are needed to perform studies to increase basic knowledge of how stem cells in the stomach differentiate, as well as to
Table 5: Corpus TX Damage Outcome Summary

<table>
<thead>
<tr>
<th>Outcome</th>
<th>TX Dose/Timing</th>
<th>24 Hours</th>
<th>3 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>Med</td>
<td>High</td>
</tr>
<tr>
<td>H/K ATPase Expression</td>
<td>N.D.</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Corpus Proliferation</td>
<td>N.D.</td>
<td>N.D.</td>
<td>↑</td>
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N.D. = No Difference
Outcomes were recapitulated in Gastrin-deficient mice and with TX without EtOH administration
aid in discovering steps and signaling pathways involved in the development of gastric cancer, the second leading cause of cancer-related deaths worldwide (40). Very few markers for GSCs have been identified, and a lack of reliable molecular drivers available for manipulating these cells has made the study of gastric cancer development challenging (see Table 1).

Here, I demonstrate through long-term lineage tracing that Bmi1 and Lrig1 are expressed in corpus and antral stem cells in the gastric epithelium. Notably, 24 hours after TX activation, individual cells were labeled throughout the epithelium, not just the proliferative zone (see Fig. 12), indicating that both Bmi1 and Lrig1 are also expressed in differentiated cells.

Interestingly, Bmi1-CreER has a more mosaic expression pattern, with only a small proportion of labeled glands occurring, while Lrig1-CreERT2 lineage tracing is fairly ubiquitous throughout the gastric epithelium. The differing expression patterns may make each CreERT driver useful in different ways. For example, the Bmi1-CreER driver can be used for studies in which an investigator may wish to examine effects of knocking out a gene in the epithelium without causing lethality to the mouse. In contrast, for studies in which gene over-expression is the goal, the more ubiquitous Lrig1-CreERT2 may be more useful. However, the shared expression of these CreERT drivers with the intestine may limit their usefulness in the stomach, as some gene manipulations in the intestine may be lethal to the mouse.

**Testing Intestinal CreERT;Rosa-Reporter Activation**

My analysis of the Bmi1-CreER and Lrig1-CreERT2 drivers in the intestine was strikingly different from that of published reports. Bmi1 and Lrig1 have been described as +4 QSC markers in studies utilizing the Rosa26-LacZ reporter strain (43, 47). In sharp contrast, analysis of both the Rosa-LacZ and Rosa-Tom reporters in my experiments yielded much more
penetrant reporter activation within the intestinal epithelium, including differentiated cells along the villi. Given the disparity, it was important for future studies in the field to determine the underlying cause of these differences.

I hypothesized that these disparities may have been due to either differences in the dose of TX administered to activate the CreERT, or the Rosa-reporter strain used to mark the CreERT-expressing cells. Therefore, the effect of varying doses of TX on the amount of reporter activation with various Rosa-reporter strains was examined. Across all four intestinal CreERT drivers tested, the Rosa-Tom reporter had a higher level of activation when compared to the Rosa-LacZ reporter. This was confirmed by morphometric analysis of the number of activated crypts in the proximal intestine. Furthermore, the Rosa-YFP reporter was not perceived 24 hours after TX activation despite the use of an anti-GFP antibody that detects the YFP. However, YFP expression could be detected three days post-treatment by GFP immunostaining. Notably, previous reports of Bmi1-CreER and Lrig1-CreERT2 utilized the LacZ and YFP reporters, which may explain in part the differing interpretation of the expression pattern (43, 47).

The reporter activation differences among Rosa-reporter strains may be due to different factors. First, at the genetic level, some reporter constructs may be easier to activate than others. For example, some LoxP sites may be easier for the Cre recombinase enzyme to access or recombine. Snippert et al. generated a multi-colored reporter allele, Rosa26-Confetti, which should express one of four fluorescent proteins in a stochastic manner (52). However, while three of the colors were expressed at near-equal ratios, one of the colors had a much lower rate of expression, indicating some underlying difference in activation within the same construct. Second, after recombination, reporter expression may vary at the transcriptional and post-transcriptional level. Differences in reporter activation may also stem from the makeup of the
reporter constructs themselves, as the same results (higher activation of Rosa-Tom compared to Rosa-LacZ and no expression of Rosa-YFP after 24 hours) were observed across all CreERT drivers tested. Both the Rosa-Tom and Rosa-YFP have a CMV-IE enhancer/chicken beta-actin/rabbit beta-globin hybrid (CAG) promoter, while the Rosa-LacZ does not (53). The Rosa-Tom allele has another enhancer, a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), which stabilizes the mRNA (30). This construct also contains a residue linker that fuses two copies of the resulting RFP together to make a tandem dimer that has a rapid maturation time and a high brightness factor of 95 (defined as the product of molar extinction coefficient and quantum yield (mM × cm$^{-1}$)) (8, 51). In contrast, the eYFP in the Rosa-YFP does not have mRNA stabilizing elements and is a weak dimer with a brightness factor of 51 (mM × cm$^{-1}$). In addition, careful studies conducted by Patterson et al. revealed that eYFP is sensitive to chloride ions and pH; at pH 6.5 the eYFP is only 50% fluorescent (41). Therefore, handling of the Rosa-YFP tissue through processing and preparing for imaging may be a factor in the ability to detect the YFP signal. Other YFP genes such as Citrine and Venus have been constructed that introduced mutations into the GFP gene to decrease sensitivity to pH and chloride ions, and increase maturation rate and fluorescence intensity (17, 35). These may more closely recapitulate the apparent increased sensitivity of the Rosa-Tom reporter if Rosa reporter strains were to be made with Citrine or Venus constructs. Furthermore, differences in the way I processed tissues for histological analysis compared to other labs may be a possible explanation for why my data with the LacZ reporter was different from published data with the Bmi1-CreER;LacZ and Lrig1-CreERT2;LacZ despite the use of the same CreERTs and Rosa-LacZ construct (43, 47).
The difference in reporter activation with each CreERT has far-reaching implications for experimental outcomes. First, as was discovered here, the choice of reporter can have a large impact on the cells that appear to be expressing the CreERT driver. Clearly, this study and others (33, 59, 60) have shown that Bmi1 and Lrig1 are expressed in differentiated cells in the intestinal epithelium as well as QSCs. This difference was most strikingly shown with the Rosa-Tom reporter, but it was also observed with the Rosa-LacZ reporter. In contrast to my studies, which examined differences in Rosa-reporter constructs with a specific intestinal CreERT driver, Li et al. (29) have also shown differences in expression patterns among specific drivers. Utilizing the QSC gene, Bmi1 as a driver either for a CreERT, or an internal GFP reporter construct, they showed that the Bmi1-GFP reporter was expressed in very few detectable cells within intestinal crypts, while the Bmi1-CreERT;Tom was expressed in cells in the crypts and the villi (29). These findings, taken with my data that different Rosa-reporters have different activation efficiencies, further illustrate that genetically-engineered CreERT or reporter constructs may not recapitulate actual gene expression patterns and may not be suitable to identify functional ISC populations unless other corroborating methods are used.

Second, expression of a reporter allele cannot be used as a surrogate for activation or deletion of other genes in the same tissues that utilize the CreERT/Lox system. The fact that various reporter alleles have different activation events with the same CreERT driver line precludes the idea that the same CreERT will activate a reporter and another allele at the same rate in the same cell. Therefore, activation or deletion of a gene that does not have an internal reporter must be confirmed by other quantitative or semi-quantitative means such as quantitative-PCR (qPCR) or by gene expression analyses such as RT- or qRT-PCR. These findings stress the
importance of carefully considering the best CreERT-reporter system for the type of experiment investigators wish to pursue.

**Spontaneous CreERT Activation**

Upon examination of vehicle-treated samples, a low level of TX-independent reporter activation was noted. This is not the first study to observe spontaneous CreERT-reporter activation in the absence of TX, as other studies have found the same phenomenon in cell lines *in vitro* (7) and in other tissues (16, 34). Notably, Schuijers et al. recently found an approximate rate of 12% non-induced crypt activation with an intestinal stem cell-specific marker, Olfm4-CreERT2 (49). Differences between non-activated reporter expression observed among the CreERT drivers tested here are difficult to interpret. Given that three of the intestinal CreERT drivers are putatively expressed in transit amplifying (TA) cells, the timing of tissue harvest can have a large impact on observations of non-induced reporter activation. Activation in TA cells will lead to ribbons of labeled cells that will migrate up the villi and be shed into the lumen within days. Therefore, the ribbons of cells observed with the CreERT drivers and Rosa-Reporters may be due to perpetual activation in stem cells, or it may be an example of a “snap shot” in time of activation in short-lived progenitor cells.

**Tamoxifen Dose, Activation, and Damage**

Three different TX doses were tested to determine if the concentration administered had an effect on Rosa-LacZ or Rosa-Tom activation. Surprisingly, in each case, it was determined that increasing the concentration of TX did not correspond to an increase in the extent of reporter labeling (Figs 5–8). These findings are in contrast to reports in other tissues in which Rosa-LacZ
activation with a CAGG-CreERT was found to be TX dose-dependent in the cerebral cortex, cerebellum, heart, kidney, and lung (16). To my knowledge, this is the first study conducted to test the effect of varying TX doses on CreERT/Rosa-reporter activation in GI tissues. Due to the fact that higher doses of TX did not increase CreERT activation, I would hypothesize that the only way to increase activation is to administer multiple doses over time, which may be useful for genetic manipulation studies.

Although higher doses of TX do not equate to more activation in the intestine, TX does cause damage to the gastric epithelium. TX is a pharmacologic drug used to combat certain cancers; therefore, testing its putatively harmful effects in gastric tissues was important. Previous reports have shown that TX can cause parietal cell death in mice at doses of 150mg/kg body weight and higher, with the height of cellular damage occurring three days post-treatment (19). However, damage to the gastric epithelium due to TX was shown to be transient, with repair to the tissue in 14 to 21 days (19). The mechanism by which TX causes parietal cell death is not well understood. It is hypothesized that TX acts as a protonophore, thereby affecting intracellular pH (19, 28), which could be deleterious to acid-secreting parietal cells.

Here I confirm that the high dose (200mg/kg body weight) of TX used causes significant damage to the gastric corpus, with decreased mRNA expression and immunostaining of the parietal cell marker H/K ATPase and increased proliferation 24 hours after administration. Interestingly, I have also demonstrated that, while the medium dose (100mg/kg body weight) of TX does not show obvious morphological damage 24 hours after treatment, at the mRNA level there is a significant decrease in the parietal cell marker Atp4a. Upon examination of tissues three days post-treatment, the gastric damage was exacerbated with the high dose, as well as the medium dose. Furthermore, by 72 hours, the low and medium doses also showed a significant
decrease in the expression of *Atp4a* and the medium dose revealed a significant increase in proliferation. These data indicate that even low doses of TX can cause gastric damage (see Table 5). It was also determined that the EtOH used to prepare the TX solution was not the cause of the damage nor was the route of administration, as TX-treatment via oral gavage did not ameliorate the effects.

Treatment of mice with the proton pump inhibitor, omeprazole, has been shown to prevent parietal cell toxicity from TX to some extent (19), indicating that inhibition of acid secretion is partially protective. Gastrin knockout (GKO) mice have reduced acid secretion (21) and the growth hormone gastrin has been shown to be required for increased proliferation resulting from a loss of parietal cells (20, 39). Thus, to determine the mechanism behind the resulting proliferation increase in response to parietal cell loss due to TX, GKO mice were treated with TX. Surprisingly, it was found that the GKO TX-treated corpus also had significantly increased EdU+ cells 24 hours post-treatment, which sustained compared to vehicle-treated corpus even after three days, suggesting that another pathway, at least in gastrin-deficient animals, is responsible for increasing proliferation after TX damage.

Taken together, these data indicate that timing, as well as dosage should be considered when undergoing experiments utilizing TX for genetic recombination in Cre/Lox systems. Indeed, differences in TX dosing and timing of examination of gastric tissues due to the transient nature of the damage may account for some of the controversy in the field, with some studies showing minimal or no TX damage and some studies showing gastric changes in response to TX (19, 45, 55). Furthermore, a recent study has found that TX can damage ISC s by inducing apoptosis (62). In response to this study, Kozar et al. tested the effects of high and low doses of TX and found no difference in stem cell numbers, therefore concluding that TX does not cause
damage to stem cells (27). Clearly, as TX use is so prevalent in the GI field, further study needs to be done to elucidate the mechanisms underlying TX toxicity to GI tissues.

**Conclusions**

The data here illustrate that the careful consideration of reporter constructs, TX dosing, and timing of tissue examination should be taken into account when undergoing experiments utilizing TX. I have shown that increasing the concentration of TX in a single dose does not increase the amount of reporter activation within the range that was tested, though it does increase gastric tissue damage. Through my studies, I have ruled out the possibility of EtOH and route of administration as the underlying causes of TX damage. I have also demonstrated that gastrin is not the mechanism of increased proliferation as a result of that damage. Importantly, I have also identified two new gastric stem cell markers, *Lrig1* and *Bmi1*. Both of these markers are expressed in the corpus and antrum and will be useful for future studies examining pathways that regulate stem cell homeostasis in the gastric epithelium. These CreERT drivers also represent the first reliable stem cell-driven CreERTs in the glandular corpus.
References


Appendix: IACUC Approval

This project was approved for the use of animals by the University Committee for the Use and Care of Animals (UCUCA) at the University of Michigan under approval number PRO00004990 from 8/20/2013 to 8/20/2016.