Chapter 2
Clonal Evolution of Stem Cells in the Gastrointestinal Tract

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The intestine is one of the fastest renewing tissues in our body. Its epithelial layer renews every 3–5 days in the adult. This rapid turnover is sustained by a small number of tissue-specific stem cells. Previous studies aimed to identify a rare population of long-lived cells by conventional means such as electron microscopy, DNA label retention, and staining with several rare cell markers [1–5]. Nevertheless, these studies were not sufficient to definitively identify the true potential of these adult stem cells in tissue homeostasis and regeneration. In 2007, Barker et al. reidentified crypt base columnar (CBC) cells as the stem cell of the intestine using elegant lineage tracing of $Lgr5^+$ cells [6]. These $Lgr5^+$ cells proliferate every day to produce a sufficient number of progenitors to fill up the pocket-like structures known as crypts (Fig. 2.1). Proliferating progenitors migrate upward while differentiating into nutrient-absorbing enterocytes as well as secretory cells that produce mucins (goblet cells) or hormones (enteroendocrine cells). These three cell types comprise the epithelium of the villus, a digit-like protrusion toward the gut lumen. Paneth cells migrate downward and stay together with CBC cells. They play a key role in the secretion of antibacterial compounds and in stem cell maintenance by providing growth factor signals (e.g., Egf, Notch, and Wnt ligands) [7].

Lineage tracing experiments have become the gold standard for investigating the longevity and differentiation potential of adult stem cells in vivo. Currently, lineage tracing experiments mostly rely on the tamoxifen-inducible Cre-Recombinase enzyme and transgenic reporter mice (e.g., Rosa26-reporter) [8–18]. In this system the Cre DNA recombinase is linked to a fragment of the estrogen receptor (ER), generating a fusion protein called CreER [19]. The Cre activity of this artificial

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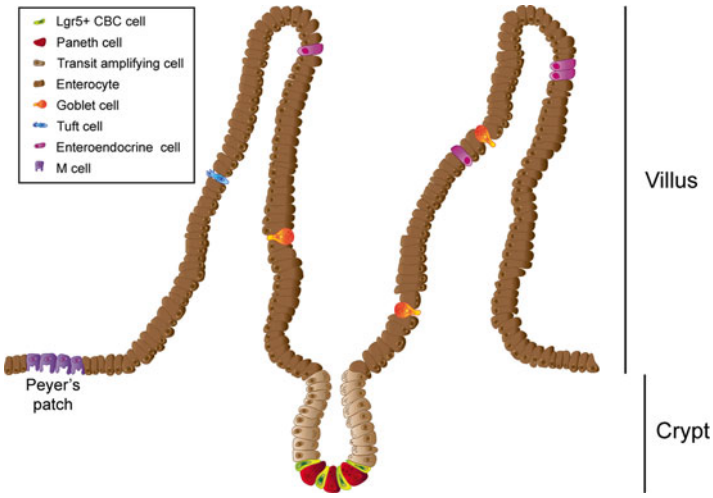

Fig. 2.1 Epithelial layer of the small intestine (adapted from Koo et al. [51]). The epithelial barrier of the small intestine is comprised of a single cell layer that forms protrusions, called villi, and invaginations, called crypts. Crypt base columnar (CBC) cells can be found in the crypt base intermingled with Paneth cells. Stem cell proliferation drives cell migration upward to replace the functional cell types within the intestinal epithelium. CBC cells mainly generate transit amplifying cells that are located just above the crypt base. During their upward migration these cells proliferate and differentiate to nutrient absorbing enterocytes, secretory enteroendocrine, or goblet cells. Microfold cells (M cells), involved in antigen presentation to the immune system, and tuft cells of unknown function are also generated during this process. Paneth cells remain in the crypt base to provide crucial niche factors for the CBC stem cell population

fusion protein is often under spatial control as it can be placed under a tissue-specific promoter. Its activity can also be regulated temporally as it enters the nucleus only if it binds to its ligand estrogen or the synthetic analog tamoxifen, which can be provided via a direct intraperitoneal injection to the mouse. Once it moves into the nucleus, CreER can facilitate recombination between LoxP sites. When combined with a Cre reporter, tamoxifen-activated CreER can excise a LoxP-flanked "stopper" cassette of the reporter to induce the expression of β-galactosidase or a fluorescent protein. Placing the CreER enzyme under the control of a stem cell gene-specific promoter, this enzyme can permanently mark a stem cell by genetically removing the stopper. Once activated, this genetic change is inherited by all progeny from the marked stem cell (Fig. 2.2).

As explained, the heart of this method lies in the identification of a specific marker gene of the target cell type [20]. The stem cell nature of CBC cells was not well understood until the Wnt target gene Lgr5 was identified as a specific stem cell marker. Employing this novel marker, lineage tracing experiments proved CBC cells are long-term adult stem cells in the gut epithelium [6]. Technically, an Lgr5-eGFP-ires-CreERT2 knock-in mouse was designed to express both eGFP and CreERT2 simultaneously under the control of the endogenous Lgr5 promoter. In this way, the

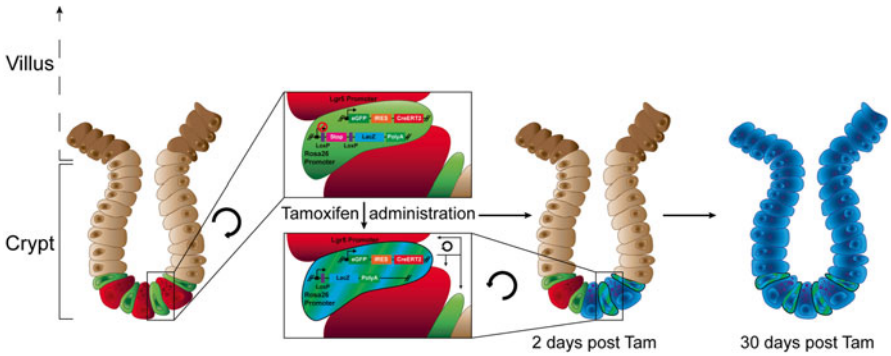

Fig. 2.2 Lineage tracing in Lgr5⁺ CBC cells of the small intestine. In a mouse model expressing eGFP and the tamoxifen-inducible CreERT2 enzyme under the transcriptional control of the Lgr5 promoter, all Lgr5⁺ cells express GFP and CreERT2. Upon tamoxifen administration the Cre recombinase relocates into the nucleus. In combination with reporter alleles (e.g., RosaR26-LacZ), the recombinase induces the expression of the reporter gene by excision of a stop signal. Subsequently, Lgr5⁺ CBC cells are labeled both with Lgr5-promoter-controlled eGFP and with the constitutively expressed reporter gene. Labeled stem cells self-renew and generate functional cell types of the intestinal epithelium without losing the reporter label, resulting in complete labeling of the entire crypt-villus axis

CBC cells could be visualized by eGFP and they also expressed tamoxifen-inducible CreERT2 in a stem cell-specific manner [6]. By administering tamoxifen to Lgr5-eGFP-ires-CreERT2;Rosa26-reporter mice, a single Lgr5⁺ CBC cell could be labeled to express a reporter gene (e.g., β-galactosidase) under the control of the constitutively active Rosa26 promoter. One day after the induction, X-gal staining revealed specific induction of β-galactosidase in cells at the crypt base. Within 5 days, the progeny from this single cell formed a longitudinal blue ribbon in the epithelium, suggesting that all cells within the ribbon are derived from a single Lgr5⁺ cell [6] (see also Fig. 2.2). Immunohistochemical analysis revealed the existence of all known intestinal epithelial cell lineages of the intestine in this ribbon [6, 21, 22]. Most importantly, these ribbon-shaped whole crypt-villus axis tracings were readily detectable at time points of more than 1 year posttamoxifen injection, proving that Lgr5⁺ CBC cells indeed represent a long-lived adult stem cell population of the intestinal epithelium [6].

Lgr5 marks not only intestinal stem cells but also stem cells in the pylorus glands of the stomach and in colonic crypts [6, 23]. Other genes that are specifically expressed in CBC cells are Tnfrsf19 (Troy) as well as Olfm4, Ascl2, and Smoc2 [24]. Troy lineage tracing experiments revealed a slowly cycling stem cell population in the gastric corpus glands [25]. With these two markers, we have now identified many endodermal adult stem cells from stomach to colon. In this book chapter, we will first describe clonal behavior of intestinal stem cells in homeostasis, regeneration, and tumorigenic alteration. We will then summarize our recent understanding of the clonal behavior of gastric stem cells. Finally, the relationship between novel Troy⁺ corpus stem cells and conventional gastric isthmus stem cells will be discussed.

How Many Stem Cells Are in the Intestinal Crypt?

The number of stem cells needs to be strictly regulated to avoid the generation of too many transit amplifying or differentiated cells within the intestinal epithelium. Lgr5$^+$ CBC cells divide once a day, generating new CBC cells which reside at the base of each gland as stem cells [26]. The location of these CBC cells deep within the pocket-like crypts is key to the tight control of the stem cell population. As there is limited space for stem cells in the crypt base, only a fixed number of stem cells can fit into this stem cell zone. Consequently, each stem cell clone competes for this limited space with other stem cell progeny and only the winning stem cell clone of this competition can occupy the whole crypt with its clonal descendants. As all stem cells initially have the same chance to be the winner, this process is described as "neutral competition." Under homeostatic conditions, the winning stem cell clone in the crypt base gives rise to all the functional cell types of the intestinal epithelium. The cellular hierarchy under these conditions is strictly regulated, so that cells that are pushed out of the stem cell niche are first committed to a transit amplifying progenitor fate before differentiating to the various terminally differentiated cell types (e.g., enterocytes, goblet cells, enteroendocrine cells, and Paneth cells).

So how many stem cells are in the crypt? Initially, the number of stem cells was deduced from the number of Lgr5$^+$ stem cells in the crypt. Using flow cytometric analysis of Lgr5$^+$ intestinal stem cells, Snippert et al. [26] carefully set the threshold of Lgr5-GFP intensity that determines a defined population of Lgr5$^+$ cells in their flow cytometry data. Based on this GFP intensity level, the authors counted the number of Lgr5-GFP$^+$ cells in the crypts of the duodenum, jejunum, and ileum. On average, each small intestinal crypt contains around fourteen Lgr5$^+$ cells. Only crypts from the ileum displayed slightly higher numbers. In this case, the authors regarded all Lgr5$^+$ cells as functional stem cells in the crypt, since Lgr5$^+$ stem cells were found to be a single population coexpressing various other stem cell markers (e.g., Olfm4, Ascl2, and Smoc2). However, the effect of crypt structure and the limited niche space were not fully taken into consideration in this analysis. A few years later, another group developed a novel strategy—continuous clonal labeling. Using this method, they noticed increasing numbers of fully labeled crypts as well as stable fractions of partly labeled crypts. With a given mutation rate, the group predicted the actual number of working stem cells in the crypt to be only 5–6 in the small intestine [27], which is much fewer than initially predicted [26]. Despite accurate modeling of stem cell behavior, all approaches that aimed at understanding how stem cell populations compete with each other are based on the analysis of multiple independent stem cell clones at various time points and the retrospective development of models that can fit the observed clonal expansion data. To catch a glimpse of the actual clonal competition within the intestinal crypts, the group of van Rheenen established a sophisticated in vivo live-imaging technology to study intestinal stem cell behavior. This revealed heterogeneous clonal behavior of Lgr5$^+$ cells, with cells located close to the center of the crypt having a much higher probability of generating a clone that could occupy the entire crypt, suggesting that

the physical position of each stem cell could be a major determinant of stem cell potential, even if each stem cell has the same biological properties [28].

What Factors Determine Stem Cell Number in the Crypt?

It is thought that mainly Paneth cells and underlying mesenchymal cells are responsible for the niche formation crucial for stem cell maintenance and regulation. Both Paneth cells and the underlying, yet unidentified, mesenchymal cells produce the most potent growth factor of the intestinal epithelium—Wnt ligands. These cells also produce other growth factors supporting Lgr5$^+$ stem cells. Currently, the Paneth cell is the best characterized support cell for Lgr5$^+$ stem cells. Paneth cells reside together with Lgr5$^+$ stem cells at the crypt base (Fig. 2.3a). The basal membrane of Paneth and stem cells shows icosidodecahedron-like geometry, where the large Paneth cells occupy pentagons and the small Lgr5$^+$ stem cells are squeezed in between the Paneth cells as triangles. This structure maximizes the shared membrane between Lgr5$^+$ stem cells and Paneth cells while minimizing isologous interactions, suggesting the importance of the heterologous interaction between stem cells and Paneth cells [7, 26]. Indeed, Paneth cells provide growth factor signals such as EGF, Wnt3, and Notch ligands [7], which have been shown to be crucial for stem cell maintenance both in vivo and in vitro.

Paneth cells have been identified to at least partially provide the niche required for stem cell regulation. Depletion of Paneth cells via loss of Sox9 resulted in Olfm4$^+$ CBC stem cell loss, illustrating the close functional relationship between Paneth cells and intestinal stem cells. Paneth and other secretory lineage precursors in the crypt express the Notch ligands Dll4 and Dll1, respectively. Removal of both ligands caused stem cell exhaustion [29]. Ablation of Wnt3 from the intestinal epithelium failed to show its importance in stem cell maintenance in vivo due to redundant Wnt ligands being secreted by the underlying mesenchyme [30]. Nevertheless, the indispensable role of Wnt3 for stem cell maintenance has been shown in Wnt3-null intestinal organoids, which were unable to survive in vitro as the organoid stem cell population, lacking alternative Wnt sources, is fully dependent on the paracrine Wnt source provided by Paneth cells [30]. Interestingly, Math1 mutation in the intestinal epithelium liberates Lgr5$^+$ stem cells from their requirement for Paneth cells and Notch activation [30–32]. However, in vitro organoid culture of Math1 mutants again proved the importance of Paneth cells, the paracrine Wnt source for intestinal stem cell maintenance [30, 32]. An accurate management of stem cells by Paneth cells was reported in normal and fasting status [33]. Calorie restriction leads to attenuation of the mTORC1 signaling pathway in Paneth cells resulting in production of cyclic ADP ribose, which in turn stimulates the self-renewal of intestinal stem cells. Taken together, while a stromal niche has an influential effect on intestinal stem cell maintenance, the Paneth cell is a major player in the generation of the epithelial niche, which delicately controls the behavior of stem cells through their close contact.

Neutral Competition and the Rules of the Game

As described earlier, in neutral competition stem cells compete for the limited niche space within the crypt. Stem cell-derived clones can undergo (1) expansion, (2) contraction, or (3) irreversible extinction (Fig. 2.3b) [34, 35]. Although every stem cell is predicted to have the same potential to win this competition, we now have evidence that stem cells residing close to the center have a higher chance of being the winner. Under homeostatic conditions, we postulate that this neutral competition among stem cells is largely affected by the physical environment rather than by biological differences among individual stem cells. For instance, the proximity to the center of the crypt base, contact area between stem cells and Paneth cells, and the strength of

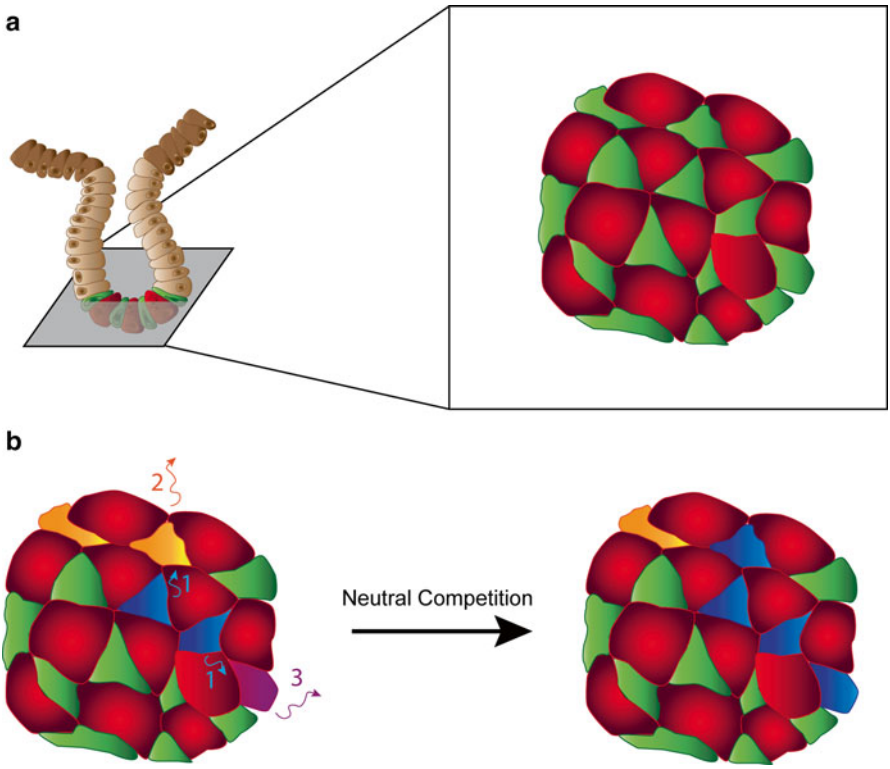

Fig. 2.3 Neutral competition illustration in intestinal crypts. Stem cells (*green*) of the small intestine are intermingled with Paneth cells (*red*) at the base of intestinal crypts (**a**). Clonal labeling illustrates the possible neutral competition-mediated outcomes for each clone (1): Expansion; (2): Contraction; (3): Extinction (**b**). The blue clone is located in the center of the crypt base tightly associated with Paneth cells whereas the orange and the purple clone are located toward the edge of the stem cell zone. Clonal expansion (1) of the blue clone results in Reduction (2) of the orange clone and Extinction (3) of the purple clone [28]

attachment to the basal matrix determine the chance of a stem cell staying within the stem cell zone. Thus, a stem cell located more centrally, with a larger area of contact with Paneth cells and basal matrix has a physically firm location that eventually proves advantageous to the stem cell toward being the victor of neutral competition (see Fig. 2.3b). However, all these physical conditions change in a dynamic manner such that a clone can only be the sole victor if it fills all stem cell niches in the crypt with its own daughter stem cells. It is important to keep in mind that the beginning of this neutral competition between stem cells is arbitrarily defined by the time point at which lineage tracing is induced. Clonal competition has occurred before this labeling event and will still continue after one clone has taken over the entire crypt.

In the neutral competition model it is assumed that all players are equally competent. However, in the actual biological context, this fair play can be biased by genetic alterations in each stem cell player. For example, tumorigenic mutations can provide a clonal advantage to stem cells. Both APC loss and K-Ras activation improve the clonal survival rate during clone competition [36, 37]. Interestingly, p53 mutation provides a similar clonal advantage only in specific contexts, such as inflammatory colitis. In other words, a stem cell having tumorigenic mutations has an advantage in filling up a whole crypt with its own daughter cells that carry the same mutation. As K-Ras activation and p53 loss alone do not cause an obvious morphological change, it is possible to accumulate these phenotypically invisible mutant cells with genetic lesions in crypts under homeostatic (K-Ras mutation) and inflammatory (p53 mutation) conditions.

What about stem cell players that are in danger of losing, or that are already out of, the competition? It is not the end for these "losers." Under specific conditions, certain cell types were shown to reacquire stem cell properties. Two special cell types have been identified by two groups: $Lgr5^+$ label-retaining cells [38] and $Dll1^+$ secretory progenitors [39]. Both cell types are not proliferative, or less proliferative than $Lgr5^+$ stem cells or other fast-dividing transit amplifying cells. These cells are committed early progenitors for the secretory lineages. Thus, they will mainly differentiate into terminally differentiated cells such as goblet cells, enteroendocrine cells, and Paneth cells. When the mouse intestinal stem cell compartment is perturbed by sublethal irradiation, rapidly dividing $Lgr5^+$ stem cells die and are quickly depleted from the stem cell zone. In this situation, both $Lgr5^+$ label-retaining cells and $Dll1^+$ secretory precursor cells can enter the stem cell zone, restore close contact with niche cells, and undergo dedifferentiation to regain stemness. Therefore, even after losing the game, an early committed progenitor can still rejoin the clonal competition as a stem cell.

These findings demonstrate not only the cellular plasticity of lineage restricted cells under tissue regeneration, but they also illustrate the importance of niche space-mediated stem cell maintenance. In this context, the limiting factor is again the number of Paneth cells providing niche space for the intermingled $Lgr5^+$ stem cell population. When all $Lgr5^+$ stem cells are depleted by γ-irradiation, a committed progenitor can enter into close contact with Paneth cells again. Niche factors from this Paneth cell are thought to help the committed progenitor to reacquire stem cell properties. Among other factors, the Wnt ligand was first found to be an important

niche factor that can allow Dll1$^+$ secretory progenitors to revert back to a stem cell fate, as sorted Dll1$^+$ cells were able to generate intestinal organoids containing Lgr5$^+$ cells if they were cultured in Wnt3a-containing media [39].

Taken together, the number of stem cells and clone dynamics in the stem cell zone of the crypt are believed to be tightly regulated by Paneth cells providing the niche space. Under normal homeostatic conditions, all stem cell players compete with the same chance to be the winner, yet the physical environment around each stem cell can affect the survival chances of individual stem cell clones. Lastly, a tumorigenic mutation can endow a significant clonal advantage to the mutant stem cell, whereas damage-induced stem cell loss may recall the losers of this competition (e.g., committed secretory progenitors) to play again as a dedifferentiated stem cell player in the game of neutral competition.

Dynamics in the Pyloric Glands of the Stomach Epithelium

The mouse stomach can be subdivided into three distinct zones. While the forestomach is comprised of a stratified epithelium, the corpus and the pylorus display a glandular epithelial organization (Fig. 2.4). Both pyloric and corpus glands can be subdivided into distinct zones: (1) pit, (2) isthmus, (3) neck, and (4) base. While glands of the pylorus consist primarily of mucous secreting cells, corpus glands show a distinct cellular composition in each of the four zones. The uppermost segment, the pit, contains mucus-secreting pit cells. The adjacent isthmus zone contains proliferative, undifferentiated cells. The next segment, the neck, is composed of mucous-secreting neck cells. The base is populated by pepsinogen-secreting chief cells. Hydrochloric acid-secreting parietal cells and hormone-secreting enteroendocrine cells are scattered throughout entire glands [40]. The highly specialized cell types that comprise the majority of pyloric and corpus glands have to be constantly replenished in order to maintain tissue function. This demand for differentiated cells requires a tightly controlled stem cell compartment at the base of the epithelial hierarchy, as previously described for Lgr5$^+$ stem cells of the intestine. Although pylorus and corpus glands are derived from the same embryonic origin, adult tissue homeostasis of the two regions appears to be differentially controlled.

For both pylorus and corpus, undifferentiated cells located in the isthmus have been proposed to represent a multipotent stem cell population that gives rise to all cell lineages of the adult epithelium [41–47]. In 2002, Bjerkens and Cheng applied a chemical mutagenesis-induced lineage tracing strategy to show that the adult gastric epithelium harbors functional multipotent stem cells [48]. In this approach, mice expressing β-galactosidase (LacZ) under control of the Rosa26 promoter are treated with the chemical mutagen *N*-ethyl-*N*-nitrosourea, resulting in mutation-mediated inactivation of the LacZ allele in some cells. If progenitor or multipotent stem cells are labeled by this approach, all their descendants will inherit the nonfunctional allele and will therefore not be labeled following LacZ staining. The group found evidence for the existence of long-term, self-renewing stem or

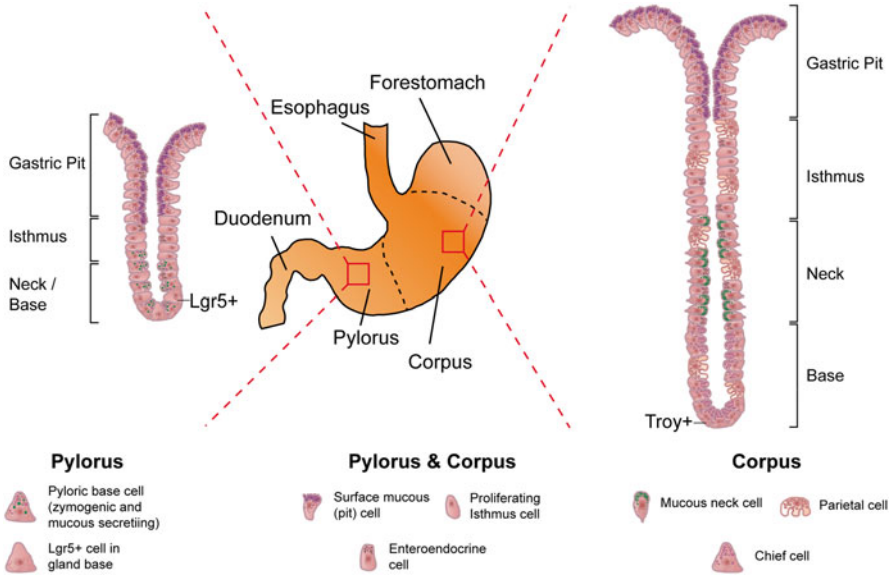

Fig. 2.4 Stomach structure and epithelial organization of the glandular corpus and pylorus. The mammalian stomach is divided into three parts: stratified forestomach and glandular corpus and pylorus. The glandular part shares a common organization, with glands being subdivided into four zones. Directly adjacent to the stomach lumen is the gastric pit, comprised of mucous pit cells. The cellular composition of the gastric pit is similar in corpus and in pylorus with the main function being secretion of mucous and subsequent protection of the stomach epithelium. Further within the gland is the isthmus zone, which harbors proliferative, granule-free, undifferentiated cells. In the corpus, at the bottom of the gland is the neck and the base, two distinct zones comprised mainly of mucous-secreting neck cells and zymogenic chief cells, respectively. In the pylorus, the zone at the base of the gland is comprised of cells that share mucous secreting and zymogenic features. Hormone-secreting enteroendocrine cells can be found in both corpus and pylorus, whereas parietal cells, responsible for the production of hydrochloric acid, are only found in the corpus. While in the pylorus $Lgr5^+$ stem cells in the gland base have been identified to be responsible for long-term tissue maintenance, in the corpus the isthmus is believed to harbor the long-term self-renewing stem cell population. $Troy^+$ chief cells, located at the corpus gland base, have been shown to respond to injury with rapid proliferation in order to regenerate the gastric epithelium

progenitor cell populations in the adult gastrointestinal epithelium. However, the exact position and identity of the tissue stem cells governing the homeostatic turnover of the entire gland were still unclear. The development of more sophisticated lineage tracing strategies using a putative marker for the pyloric stem cell was necessary to address this problem.

In 2010, the group of Hans Clevers was able to show that in the pylorus of the adult stomach, $Lgr5^+$ stem cells, residing at the bottom of gastric units, are responsible for long-term maintenance of the epithelium [23]. Long-term lineage tracing experiments revealed that $Lgr5^+$ stem cells are responsible for the homeostatic tissue turnover of the pylorus. In these experiments the clone size of $Lgr5^+$ stem cell-derived progeny was analyzed at various time points after labeling. Directly (2d)

after induction of lineage tracing, only Lgr5$^+$ cells in the base of glands are labeled. These labeled clones expand in the following days and result in fully labeled gastric units 10 days postinduction, highlighting the role of Lgr5$^+$ stem cells in short-term tissue homeostasis. Samples were taken up to 620d after the induction of lineage tracing and fully labeled gastric units could be readily observed, so proving the long-term self-renewal potential of the Lgr5$^+$ stem cell population in the pylorus. The combination of traditional lineage tracing strategies with mathematical modeling approaches allowed scientists to generate hypotheses about the exact mechanism of adult stem cell-mediated tissue homeostasis. As with Lgr5$^+$ cells in the small intestine, the Lgr5$^+$ stem cell population of the pylorus follows a neutral competition model for long-term self-renewal of the Lgr5$^+$ population rather than stringent asymmetric cell division-mediated self-renewal of each individual stem cell [49]. In this model, Lgr5$^+$ pyloric stem cells constantly self-renew and excess numbers of stem cells compete for the restricted niche space at the base of the glands. Consequently, over time, only one clone can survive and occupy the entire niche space of an individual gland. At this point this stem cell clone will occupy the entire axis of the gland with its own progeny. However, due to the joint restrictions of niche size and gland structure, the total number of stem cells does not increase indefinitely.

Interestingly, single Lgr5-expressing cells can form long-lived gastric organoids when plated into a 3-dimensional matrix and cultured in medium containing EGF, Noggin, R-spondin1, Wnt, and Fgf10. Under these culture conditions the Lgr5$^+$ stem cell population maintains its self-renewal potential and can generate various cell types of the gastric epithelium as shown by the expression of marker genes for chief cells and mucous neck cells (Gastric Intrinsic Factor, Pepsinogen-C, or Muc6). Slight changes of the culture conditions can direct differentiation toward Muc5ac-expressing pit cells, Periodic Acid Shift (PAS)- and Tff2-expressing mucous neck cells, and immature Chromogranin A-expressing enteroendocrine cells, suggesting that the cultured pyloric Lgr5$^+$ stem cell retains its multipotency as well as its self-renewal activity. In terms of population dynamics, this culture system suggested an intriguing aspect of the adult pyloric Lgr5$^+$ stem cells, in that these stem cells can self-renew indefinitely, albeit in vitro, when there is no restriction of niche components. This suggests that pyloric stem cells, as well as other gut stem cells from the intestine and colon, have no intrinsic limit to the number of cell cycles. Moreover, the culture conditions (e.g., basement matrix and growth factors) define the absolute niche requirement for this type of stem cells. Harnessing this unlimited self-renewal activity of adult stem cells will enable us to cultivate a large amount of adult stem cells for future cell-based therapy.

Clone Behavior in Corpus Glands of Stomach Epithelia

Despite the shared embryonic origin of the corpus and pylorus, Lgr5$^+$ stem cells could be found in the corpus only up until early postnatal stages and so appear to play no significant role during homeostasis of the adult tissue [23]. As described earlier and

similar to the pylorus, the gastric units of the corpus can be divided into pit, isthmus, neck, and base regions. Nevertheless, in contrast to the pylorus, the neck region of corpus gastric units displays a very different cellular composition, with multiple parietal cells and mucous neck cells physically separating the Isthmus from the base. Additionally, cycling cells can only rarely be found in the base of corpus glands, whereas Lgr5$^+$ cycling cells are a common feature of gastric units in the pylorus. Early labeling studies in combination with the description of cycling, immature cells located in the Isthmus lead to the assumption that the stem cell population responsible for tissue homeostasis of the corpus is located in the Isthmus zone [44–46, 48]. Nevertheless, the lack of a definitive marker of this putative stem cell population and the limitations of the chosen tracing strategies have hindered the exact identification and the conclusive proof of long-term self-renewing, multipotent stem cell populations.

In 2013, Stange et al. identified Tumor Necrosis Factor Receptor Superfamily Member 19 (Tnfrsf19 or Troy) as a potential marker that closely follows the expression pattern of Lgr5 in the small intestine [25]. In the corpus of the stomach, but not in the pylorus, Troy$^+$ cells have been identified in the base of gastric units. In this location Troy$^+$ cells were shown to be either chief or parietal cells. Lineage tracing analysis of both Troy$^+$ and chief cells revealed that Troy$^+$ chief cells but not Troy$^+$ parietal cells possess the ability to slowly repopulate entire glands, so highlighting their role as a reserve stem cell population. Labeled clones were shown to consist of all the epithelial cell types found in the corpus, illustrating the differentiation potential of Troy$^+$ stem cells. Additionally, labeled glands persisted in the epithelium for at least 1.5 years after the induction of lineage tracing, clearly illustrating the long-term self-renewing characteristics of this newly identified reserve stem cell population.

This study further emphasized how certain "fully differentiated" cells have a higher cellular plasticity than originally assumed (see also previously discussed label-retaining Lgr5$^+$ or Dll1$^+$ secretory precursor cells). Troy$^+$ stem cells share their primary role with other chief cells—pepsinogen production. Moreover, the turnover of the gastric isthmus and pit regions is very fast, supporting the idea of additional multipotent stem cells around the isthmus region. In support of this hypothesis, these slowly cycling Troy$^+$ stem cells can react to 5-fluorouracil-mediated depletion of proliferative isthmus cells with increased proliferation and rapid gland repopulation. Based on this observation, Troy$^+$ corpus stem cells were termed to be reserve stem cells in the gastric corpus unit (Fig. 2.5). Unfortunately, the exact identity of the predicted isthmus stem cells is yet to be determined. Sox2-lineage tracing experiments have demonstrated the existence of multipotent stem cells that do not exhibit any chief cell characteristics [50]. If there are two or even more stem cell populations in the gastric corpus gland, it will be interesting to understand how multipotent stem cell populations located in the Isthmus and Troy$^+$ reserve stem cell populations located in the base act together to govern tissue homeostasis and injury response of the gastric epithelium.

Like the Lgr5$^+$ pyloric stem cells, single Troy$^+$ chief cells are able to give rise to gastric organoids when cultured under specific culture conditions [25]. These Troy$^+$ stem cell-derived gastric corpus organoids contain multiple other corpus epithelial

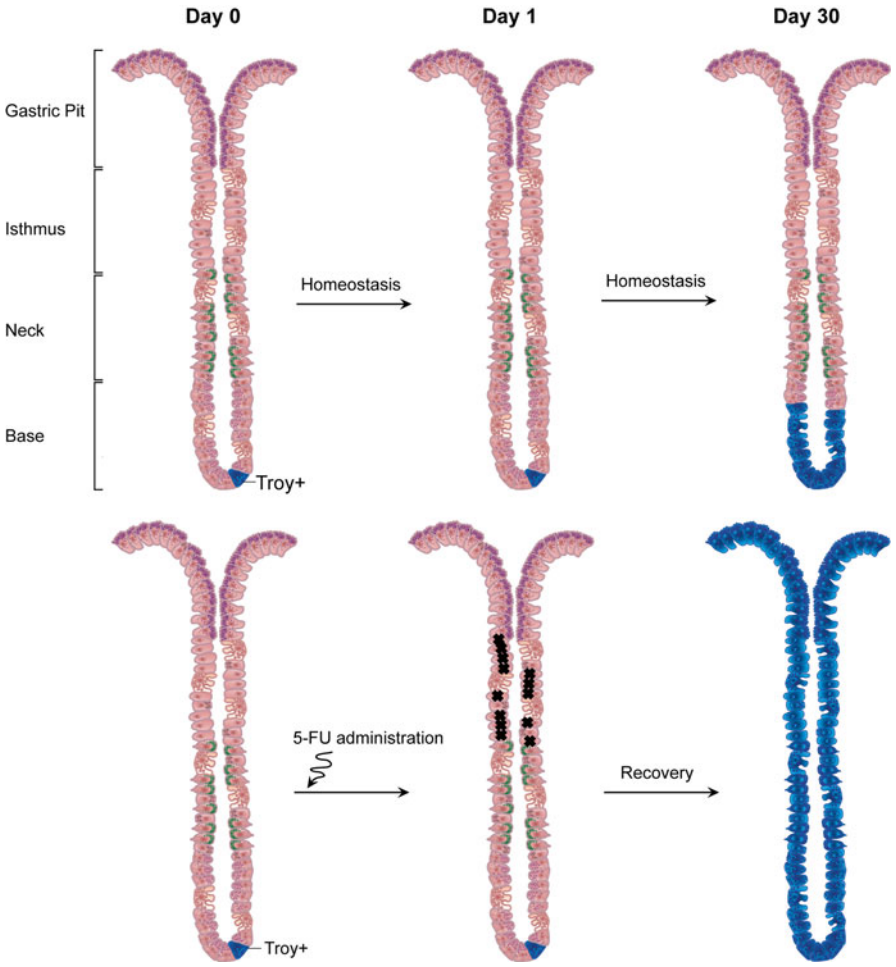

Fig. 2.5 5-FU-mediated activation of reserve stem cells in the corpus. Troy$^+$ chief cells (genetically labeled by lineage tracing at d0 in *blue*) are long-lived, fully differentiated cells located at the base of corpus glands. These cells have been shown to represent a reserve stem cell population that is mainly quiescent during homeostasis (*top panel*), but that can be reactivated if proliferative cells of the isthmus are experimentally depleted by 5-FU administration (*bottom panel*). Under these conditions Troy$^+$ reserve stem cells start cycling and generate all the cell types of the corpus gland within several weeks

cell types (mucous neck cells and pit cells) under various culture conditions, suggesting a well-retained multipotency. However, unlike their quiescent counterpart in vivo, cultured Troy$^+$ cells proliferate rapidly while maintaining Troy expression as well as chief cell characteristics. This implies that Troy$^+$ stem cells also have no intrinsic limit to their proliferation. The quiescent behavior of Troy$^+$ stem cells in vivo must be due to an unknown niche signal. By relieving this restriction, we can

now culture this interesting type of adult stem cells for many passages in vitro. Alternatively, if there is no such repressive signal in vivo, then one of the growth factors in the specific culture medium might be an activating factor for this otherwise quiescent stem cell population. Studying the exact molecular nature of the switch of this stem cell behavior (quiescence vs. active cell cycle) will help to understand the complex clone dynamics in the corpus gland of the stomach.

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