Responses of gastric epithelial stem cells and their niche to *Helicobacter pylori* infection

Jonas Wizenty[1,2], Frank Tacke[1], Michael Sigal[1,2]

[1]Department of Hepatology & Gastroenterology, Charité – Universitätsmedizin Berlin, Berlin, Germany; [2]Department of Molecular Biology, Max Planck Institute for Infection Biology, Berlin, Germany

Contributions: (I) Conception and design: J Wizenty, M Sigal; (II) Administrative support: M Sigal, F Tacke; (III) Provision of study materials or patients: All authors; (IV) Collection and assembly of data: J Wizenty, M Sigal; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Michael Sigal. Department of Hepatology & Gastroenterology, Charité – Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin, Germany. Email: michael.sigal@charite.de.

Abstract: *Helicobacter pylori* (*H. pylori*) are gram-negative bacteria that are able to colonize and persist in the stomach. Gastric cancer is tightly linked to chronic infection with this bacterium. Research over the last decades has illuminated the molecular interactions between *H. pylori* and host cells. It is now well established that *H. pylori* have multiple sophisticated means to adhere to epithelial cells and to manipulate their behavior. This interaction with the epithelium can lead to altered cell signaling, DNA damage and aberrant epithelial immunity. *H. pylori* are known to colonize the mucus layer of the stomach and surface epithelial cells. In addition, it has recently become clear that they can also penetrate the glands and directly interact with specialized epithelial cells deep in the glands. Understanding the biogeography of infection is important because gastric epithelial glands are composed of various types of short-lived differentiated cells that are constantly regenerated by a limited pool of long-lived stem cells located in base of gastric glands. Recent advances in gastric stem cell research not only led to identification of stem cell populations using specific markers but has also uncovered specific regulatory pathways and principles that govern gastric stem cell behavior and regeneration. Particularly, the stem cell state is largely dependent on signals from the niche cells that surround the stem cell compartment. The subpopulation of *H. pylori* that colonizes in the stem cell compartment triggers specific inflammatory responses and drives epithelial pathology. Colonization of gastric glands induces responses of the stem cell niche, simultaneously enhancing the cell turnover kinetics and driving the formation of antimicrobial cells in the gland base. These data reveal the high plasticity of the epithelium and its ability to adapt to the environment, which is necessary to regenerate and counterbalance infection, but simultaneously lays the grounds for development of gastric pathology and carcinogenesis.

Keywords: Antimicrobial defense; *Helicobacter pylori* (*H. pylori*); plasticity; stem cells; stomach

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The anatomy of the stomach

The stomach, as part of the gastrointestinal tract, is an intraperitoneal, muscular, hollow organ located on the left side of the upper abdomen. The gastric mucosa contains a single-layered mucin-generating surface epithelium and specialized cells forming tubular glands. The glands can be divided into the base, the isthmus and the pit facing the lumen (*Figure 1*). Corpus glands contain short-lived pit and neck mucus cells for mucus production; longer-lived parietal cells, which secrete hydrochloric acid; different hormone-producing enteroendocrine cells, and gastric chief cells, which release pepsinogen and gastric lipase (1,2). In contrast, antral glands have deeper pits and contain fewer

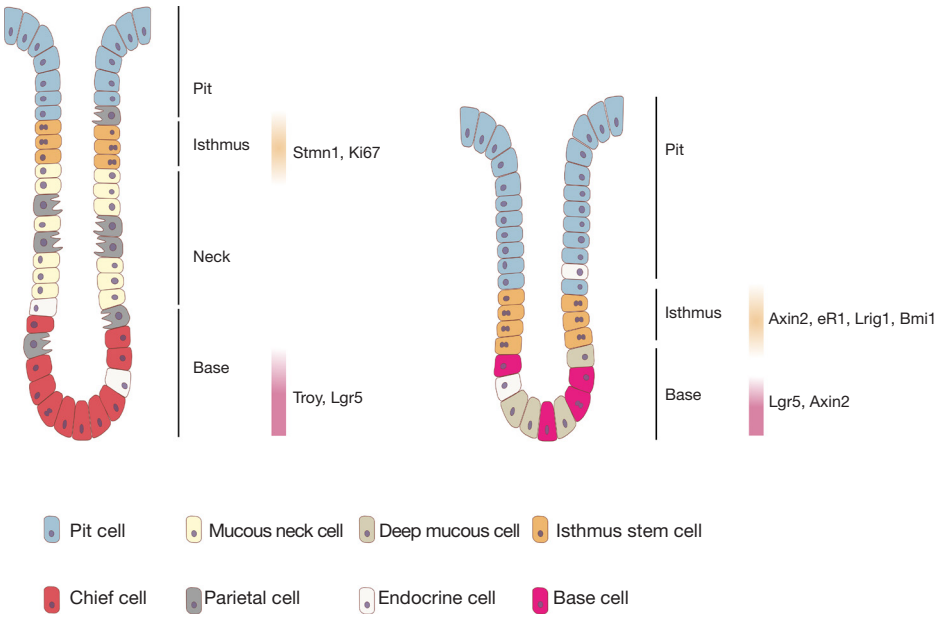

Figure 1 The glandular organization of the stomach. The stomach can be divided into two anatomical regions, the corpus (left) and the antrum (right). Both contain stem cell populations, located in the isthmus and the base.

differentiated cell types compared to corpus glands, such as surface mucous cells, deep mucous cells, enteroendocrine and tuft cells (1,3). The gastric epithelium is permanently regenerated by a small population of long-lived, dividing stem cells located in the gland itself, which are responsible for constant gland turnover. The turnover kinetics appear to be more rapid in the antrum than in the corpus (4). In general, surface epithelium cells survive only few hours or days, before being shed into the lumen, whereas some cells, such as parietal cells are more long-lived and can survive for weeks or months (5).

Gastric stem cells

Gastric glands are considered monoclonal units originating from self-renewing adult stem cells (6,7). The identification of molecular stem cell markers was recently facilitated by novel lineage tracing techniques in genetic mouse models. These models have significantly expanded our understanding of the gastric gland homeostasis in health and disease (8).

In the antrum, similarly to other parts of the gastrointestinal tract, the leucine-rich repeat-containing G-protein coupled receptor 5 (*Lgr5*) has been shown to mark a cell population in the gland base that repopulates full glands. Approximately 30% of Lgr5+ cells are actively cycling. *In vivo* lineage tracing revealed that the entire gland can be repopulated from the Lgr5+ cell compartment with appearance of various differentiated cell markers, establishing *Lgr5* as an antral stem cell marker (9). The Wnt target gene *Axin2* also marks Lgr5+ base cells and further expands to a more rapidly proliferating Lgr5-negative cell population in the lower isthmus. Lineage tracing using *Axin2* reporter mice revealed that Axin2+/Lgr5+ cells repopulate the gland more rapidly than Lgr5+ cells. Even upon loss of Lgr5+ cells, the remaining Axin2+ cells repopulate entire glands, including new Lgr5+ cells, within seven days (10).

Further markers have been used to mark isthmus cells in the stomach and it has been shown that they are distinct from gland base Lgr5+ cells. Muscle, intestine and stomach expression 1 (*Mist1*) was shown to mark multipotent progenitors in the isthmus of antrum and corpus (11,12). In addition to *Mist1*, sex determining region Y-box 2 (*Sox2*), cholecystokinin 2 receptor (*CCK2R*) and leucine-rich alpha-2-glycoprotein 1 (*Lrig1*) have been used as markers of antral and *Sox2* and *Lrig1* also of corpus stem cells (13-15). Another recently introduced isthmus stem cell marker in the antrum and the corpus is B cell-specific Moloney murine leukemia virus integration site 1 (*Bmi1*), which has regeneration potential after irradiation or gastric ulcer formation (16). Moreover, a Runt-related transcription factor 1 (*Runx1*) enhancer element, eR1 was found to be expressed in the

isthmus stem cells in antrum and corpus and in a smaller number in basal chief cells (17).

It has been shown that corpus gland base cells are marked by *Troy*. These cells are quiescent, differentiated chief cells, but they are able to act as 'reserve' stem cells and increase their proliferative activity upon injury (18). Later it was confirmed that *Troy* overlaps with a Lgr5+ subpopulation of chief cells that regenerate entire glands upon injury (19). *Lgr5* exclusively labeled 40% of gastric intrinsic factor (GIF) expressing chief cells. Lgr5-GFP+ chief cells also expressed other stem markers including *Mist1* and *Sox2*. However, in contrast to *Lgr5*, those markers were expressed in broader compartments throughout the gland (19). In agreement with the *in vivo* data, Troy+ and Lgr5+ chief cells could be cultured to generate long-lived gastric organoids (18,19).

The exact hierarchy of the cell types summarized above remains elusive and it is likely that the presented genes mark at least partially overlapping cell populations. The isthmus appears to be a critical, highly proliferative stem cell compartment in the stomach, whereas gland base cells that express *Lgr5* are rather slow cycling and show features of differentiation. Since Lgr5+ cells have been found to consist of several subpopulations (20), it will be important to understand whether the truly differentiated secretory cells in the base occasionally de-differentiate to repopulate the glands or whether Lgr5-lineage tracing data result from a partial overlap of *Lgr5* expression with more proliferative isthmus cells. Concerning the corpus, a recent report by Han *et al.* based on clonal data and single-cell profiling demonstrated elegantly the compartmentalization into two independent long-lived zones with basal, slow-cycling Troy+ and Lgr5+ stem cells and rapidly cycling isthmus Ki67+ and Stathmin1+ (Stmn1+) stem cells (21). Besides rapid vertical expansion, isthmus stem cells showed a slow drift towards clonality via lateral expansion regulated by intercalating parietal cells that act as physical barriers, and not by stem cell competition alone. Of interest, some of the suspected stem cell markers *Sox2*, *Runx1*, *Lrig1*, *Mist1*, and *Bmi1* showed a very broad expression pattern in single cell RNAseq data (21).

It should further be noted that epithelial stem cell hierarchies appear to be context-dependent and that gastrointestinal epithelia in general appear to have high plasticity, with post-mitotic, differentiated cells maintaining the ability to dedifferentiate or transdifferentiate (22). In the small intestine and colon, this high plasticity is well explored, demonstrating that nearly every cell is able to take over stem cell functions (23-25) and it will be important to further explore how acute and chronic stomach injury alters epithelial hierarchy.

Stem cell niche factors support gastric stem cells

Although stem cells have a distinct location and phenotype, their identity and behavior are largely controlled by extrinsic factors from the stem cell niche, i.e., the local microenvironment surrounding the stem cell compartment (26). Various cells, such as subpopulations of neighboring epithelial cells, stromal myofibroblasts, vascular cells, nerves and immune cells constitute the stem cell niche. The notion that the niche is the determining factor controlling the stem cell state derives from studies in mice in which stem cells were depleted. In the antrum, depletion of Lgr5+ cells lead to a rapid repopulation of gland bases that re-acquire the properties of lost Lgr5+ cells within a short period of time (10). Of note, depletion of Lgr5+ cells in the corpus does have an impact on gland physiology, at least in the long-term, suggesting that in the corpus recovery of Lgr5+ cells upon loss is less robust than in the antrum (19).

While these data have suggested the importance of the stem cell niche, more recent studies have provided molecular insights characterizing signals that are required to shape the stem cell compartment. As described above, expression of the stem cell markers *Lgr5*, as well as *Axin2* and *Lrig1*, requires canonical Wnt signaling, which is active in the gland base (9). The role of Wnt signaling in the stomach has been reviewed recently (27). R-spondin 3 from stromal myofibroblasts, specifically in the base of the stem cell niche, has been demonstrated to be essential for fully active Wnt signaling in the stomach, stem cell identity and epithelial turnover in the antrum (10). While expression of the Wnt target genes *Lgr5* and *Troy* in the corpus gland base implies active Wnt signaling in this compartment, a detailed spatial mapping of Wnt ligands has not been performed for the corpus.

A critical role of *Wnt5a* deriving from innate lymphoid cells for development of gastric cancer in the corpus has been demonstrated, proving evidence for the contribution of non-canonical Wnt signaling in gastric pathology (11). In addition, a recent report has demonstrated that different Wnt target genes are differentially expressed in Lgr5+ cells in the corpus upon tamoxifen-induced injury (19).

In addition to Wnt signaling, Notch is an important signaling pathway in the gastrointestinal tract, which stimulates stem cell proliferation via activation of the NOTCH1 (N1) and NOTCH2 (N2) receptors in the

antrum as well as in the corpus (28,29). Most likely, this pathway functions via direct ligand-receptor interaction between neighboring epithelial cells, since both ligand and receptor are membrane-bound. A detailed review of Notch signaling has recently been published (30). So far, the gastric Notch ligands and their cellular origins remain elusive. However, the functions of Notch receptors have been investigated. Notch inhibition, either globally using a pan-Notch inhibitor or by specific inhibition of N1 and/or N2, disturbed stem cell proliferation *in vivo* and in organoids. Further, Notch inhibition leads to an expansion of differentiated cells. In the corpus, an increase only of mucous neck-zymogenic lineages were described, while in the antrum mucous and endocrine cells are increased, probably due to the shorter lifespan of cells in the antrum. Conversely, Notch activation yields profound proliferation and reduced differentiation (28,29). In the antrum, Notch activates proliferation of Lgr5+ stem cells and decreases differentiation (31). Inhibition of Notch receptors results in reduced proliferation of antral Lgr5+ stem cells (29). In the corpus, N1 and N2 are expressed by isthmus stem cells, while only N2 is also expressed in the gland base. Both receptors are thought to have an additive function regarding regulation of proliferation (28). Although Wnt and Notch have partially overlapping targets, the exact interplay between both pathways in the stomach is not fully understood.

Bone morphogenetic protein (BMP) signaling also regulates gastric epithelial cell growth and differentiation. Binding of BMP to its receptors leads to the activation of Smad proteins, which enter the nucleus and regulate gene expression. In the stomach BMP-4 stimulates expression of the H+/K+-ATPase α-subunit in parietal cells and enhances gastric acid production, suggesting that BMP signaling induces differentiation into parietal cells (32). Overexpression of Noggin, a secreted factor that inhibits BMP signaling, causes loss of parietal cells and activates proliferation and expansion of transitional cells expressing markers of mucus neck-zymogenic lineages [trefoil factor 2 (Tff2), mucin 6 (Muc6), Griffonia simplicifolia lectin II (GSII)] (33). Noggin thereby induces extracellular signal-regulated kinase (ERK) activation, which contributes to the hyperproliferative state, while loss of parietal cells leads to reduced acid secretion and hypergastrinemia (33). Moreover, it has been demonstrated that inhibition of BMP signaling drives an expansion of Lgr5+ cells (34).

While these studies provide insights into a role of BMP signaling in stem cell regulation, they mostly rely on exogenous manipulation of signaling pathways, such as overexpression of Noggin, which does not show a high level of expression in the murine stomach (own unpublished data). Therefore, it will be important to further explore how the BMP pathway is regulated in the stomach in the context of homeostatic gland turnover and during epithelial injury.

H. pylori initiates changes in gastric epithelial turnover and leads to epithelial pathology

The gram-negative, spiral-shaped bacterium *Helicobacter pylori* (*H. pylori*) colonizes about 50% of the world's population. *H. pylori* colonizes exclusively the gastric mucosa, usually per oral transmission in early childhood, and persists for life, withstanding gastric acid and immune surveillance (35-38). In most cases colonization with *H. pylori* remains asymptomatic; however *H. pylori* induces an active gastritis and is the main risk factor for development of gastroduodenal ulcers and, as a long-term complication, gastric cancer (39). The WHO classifies *H. pylori* as a class 1 carcinogen. Gastric cancer causes more than 700,000 deaths per year worldwide. Until now a successful vaccine is not available, probably due to bacterial mechanisms of immuno-evasion allowing persistence of *H. pylori* in its niche (40).

H. pylori survives in the lumen of or in close proximity to gastric glands, where the mucus protects it from the low gastric luminal pH (*Figure 2*). *H. pylori* uses a complex motility and chemotaxis system to reach the gastric epithelium. It orientates with four chemoreceptors, namely transducer like proteins (TlpA, TlpB, TlpC, and TlpD) sensing numerous signals such as urea, amino acids, and metals (36,41). Chemoreceptors activate a signal transduction with the Che family that controls the flagellar direction, while the flagellar motor is controlled by the Mot family (42,43). Chemotaxis is important to sense the epithelium and to establish gland colonization, allowing *H. pylori* to directly interact with stem cells, while Che^- mutants are not able to reach stem cells (44,45). Once colonized, multiple bacterial adhesins including blood group antigen binding adhesion (BabA) (46), sialic acid binding adhesion (SabA) (47), Helicobacter outer membrane protein Z (HopZ) (48), outer inflammatory protein A (OipA) (49) and the adherence associated lipoproteins A and B (AlpA/B) (50) contribute the attachment of *H. pylori* to the epithelium, which is important for the ability of *H. pylori* to manipulate cell behavior either via direct injection of virulence factors or via secretion of toxins (51).

Among the virulence factors, the cag pathogenicity island

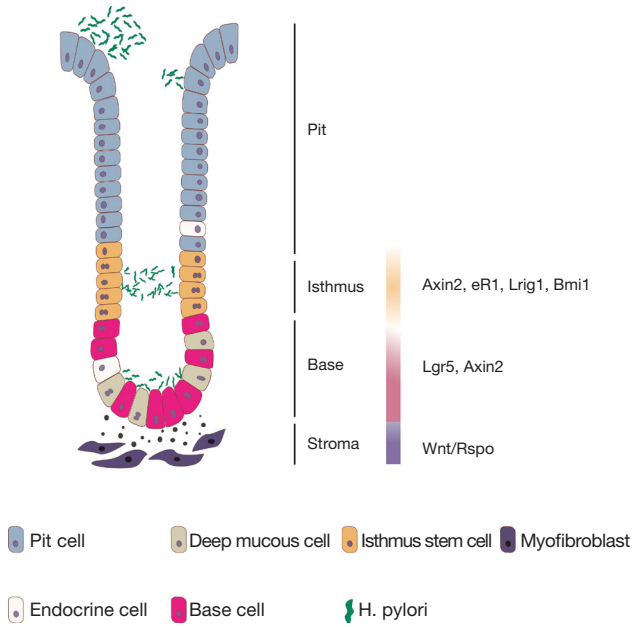

Figure 2 *H. pylori* infection of the antral gland. *H. pylori* are able to colonize the gastric gland with a minor subpopulation even reaching the gland base, where Lgr5+ stem cells reside. Upon *H. pylori* infection, R-spondin 3 from stromal myofibroblasts expands the stem cell compartment.

(cagPAI) that encodes for the type IV secretion system (T4SS) and cytotoxin-associated gene A (CagA) represents the most prominent and well-studied system. T4SS allows the injection of CagA protein into infected cells, inducing a variety of cellular responses. CagA also allows bacteria to extract nutrients from gastric cells, for example it transfers transferrin receptors from the basolateral membrane to the apical surface where the bacterium locates. Subsequently, *H. pylori* acquires iron from the host (52). Low host iron levels increase colonization of gastric glands as well as the number of T4SS pili enhancing CagA signaling (53). Both, experimental and clinical data have linked CagA to gastric cancer (54,55).

New insights have revealed that in addition to CagA, ADP-glycero-β-D-manno-heptose (ADP heptose), a small carbohydrate precursor molecule of lipopolysaccharides (LPS) synthesis, is also released into the epithelium via the T4SS. There it binds the alpha-protein kinase 1 (ALPK1) receptor, leading to activation of TRAF-interacting protein with forkhead-associated domain (TIFA) followed by NF-κB activation and the expression of pro-inflammatory target genes such as IL-8 (56-59), establishing a new CagA-independent function of the T4SS.

In addition to aberrant signaling events, direct genotoxic effects of *H. pylori* have been proposed. By direct contact with epithelial cells, stabilized by the above-mentioned adhesion molecules, *H. pylori* has been shown to induce DNA double-strand breaks (DSBs) (60). Later it was described that infection with *H. pylori* containing a functional cagPAI impairs DNA damage response (DDR). This leads to DNA damage preferentially in telomere-proximal, actively transcribed regions. Those susceptible genomic regions show overlaps with gastric cancer genomic aberrations, indicating that some genomic events in gastric cancer could be directly caused by *H. pylori* infection (61). Moreover, it has been shown, that in stomachs of *H. pylori*-infected patients, Lgr5+ stem cells show DNA damage linked to oxidative stress (62).

Given the fact that *H. pylori* can directly induce DNA damage and that it directly interacts with gland base cells in humans and mice, it will be important to address to what extent stem cell DNA damage is driven by direct effects of bacteria versus by more indirect effects such as inflammatory responses. Furthermore, a link between DNA damage and accumulation of mutations in the context of *H. pylori* has to be further substantiated.

In addition to inducing aberrant cellular signaling via T4SS, a direct interaction of epithelial cells with *H. pylori* has been shown to modulate immune responses to infection. One key mechanism to evade the immune response is provided by the bacterial factors vacuolating cytotoxin A (VacA) and γ-glutamyl transpeptidase (GGT), which suppress T helper cell activation along with an increase of a regulatory T cells (Tregs) (63,64). A recent report demonstrates an additional mechanism of how direct interaction between epithelial cells and *H. pylori* may be relevant for immune evasion: *H. pylori* cholesterol-α-glucosyltransferase (CGT), which extracts cholesterol from the epithelial surface upon attachment, leads to a disturbance of lipid rafts that are required for signal transduction of various proinflammatory cytokines such as IFNγ, IFNβ, IL-6 or IL-22 (65). In this way *H. pylori* prevents the activation of pro-inflammatory pathways in epithelial cells, thereby blocking epithelial self-defense mechanisms such as secretion of antimicrobial proteins including human beta defensin 3 (hDB3), which has been shown to be induced by IFNγ and to be highly efficient in killing *H. pylori* (66).

Together, these data illuminate the critical role of direct interaction of *H. pylori* with epithelial cells, both for

creating a protective niche for the bacteria and for inducing cascades that result in gastric disorders. Of note, most of the reports that are summarized above did not take into consideration the aspects of epithelial turnover dynamics that are discussed in the first part of the review, such as the different survival times of cells within a gland. This appears important, as injury of long-lived stem cells might have different impact on the tissue integrity than injury of a cell that will be shed into the lumen within the next hours or days. In addition to consequences of injury, it is important to address whether the biogeography of infection is linked to specific immune responses. Indeed, it has been demonstrated that undifferentiated organoid cultures appear to induce stronger pro-inflammatory responses to *H. pylori* than differentiated cultures (67,68).

Despite this, for many years the biogeography of *H. pylori* infection has not been extensively studied. We have been able to apply confocal microscopy and 3-dimensional image reconstruction to demonstrate that indeed a subpopulation of *H. pylori* are able to colonize the base and isthmus of gastric glands in a mouse model, as well as in human samples. Our techniques allowed a quantification of gland associated *H. pylori*, showing that approximately half of antral glands were colonized with *H. pylori* (44). Inside the gland *H. pylori* preferentially formed microcolonies in the isthmus, containing proliferating progenitor cells, while some bacteria also colonized the glands base (44,69) (*Figure 2*). In the proliferative zone, bacteria were found in direct contact with mitotic progenitor cells forming extracellular microcolonies directly at epithelial tight junctions. The importance of gland colonization compared to luminal surface colonization for the induction of pathologic changes was demonstrated in C57Bl6 mice from different vendors, where *H. pylori* in mice from one vendor only colonized antral glands, but not corpus glands, whereas in mice from the second vendor glands were colonized in both, corpus and antrum. It is not entirely clear which environmental factors controlled the differences in gland colonization of the corpus, but after 2 months of infection pathology was observed in sites where gland colonization occurred: while all mice showed antral pathology, only mice with corpus gland colonization showed corpus pathology.

Lineage tracing from cells derived from Lgr5+ stem cells along with hyperplasia was locally accelerated in infected glands compared to uninfected glands in the same animal or in uninfected mice. Thereby the number of bacteria in individual glands correlated positively with the lineage tracing kinetics, indicating that gland-associated *H. pylori* induced gland turnover. These effects were not observed when using bacteria that are unable to colonize gastric glands and were restricted to the surface mucus due to a mutation in the chemotaxis machinery, demonstrating that the gland turnover is induced specifically by gland-associated bacteria. Of note, *H. pylori*-induced stem cell activation is dependent on a functional T4SS system, as mice with a defective T4SS have reduced lineage tracing and less hyperplasia compared to WT mice. In addition to induced gland proliferation, the stem cell numbers significantly increased after 2 months of *H. pylori* infection (44).

To further explore the mechanisms of stem cell expansion upon infection, the regulation of Wnt signaling, being the key component for induction of stem cell associated genes, has been investigated in detail. In contrast to the small intestine, where Paneth cells have been shown to provide Wnt ligands to the stem cell compartment, Wnt ligand expression in the antrum was rather broad and none of the Wnt ligands was found exclusively in the gland base. In contrast, R-spondin 3 was found to be specifically expressed in myosin heavy chain (Myh11+) myofibroblasts surrounding the gland base and it has been demonstrated that the expression of R-spondin 3 is increased upon *H. pylori* infection, driving the expansion of stem cells and resulting in epithelial gland hyperplasia (10) (*Figure 2*). Depletion of R-spondin 3 in Myh11+ cells leads to a loss of stem cells, and infection of such mice with *H. pylori* does not lead to the expansion of stem cells observed in WT mice. In addition to its effect as a mitogen driving an expansion of Axin2+ cells, R-spondin 3 is required for differentiation of gland base cells that co-express Lgr5 as well as markers of differentiated cells such as Pepsinogen C and Gif. These cells are able to counterbalance gland colonization by secreting antimicrobial factors such as intelectin-1 (Itln1) and regenerating family member 3 gamma (Reg3g) upon infection. Itln1 from Lgr5+ cells is able to bind and agglutinate *H. pylori* in a Ca^{2+}-dependent manner, impairing its motility (20).

While such responses counterbalance gland colonization, some bacteria are able to persist in the stomach. A recent study has explored colonization dynamics of *H. pylori* in the glands using differentially labelled *H. pylori* and it has been demonstrated that once bacteria occupy a gland, other incoming strains of *H. pylori* lack the ability to colonize the same gland (70). It will be important to explore whether epithelial antimicrobial responses to gland associated *H. pylori* contribute to this colonization resistance, therefore providing a competitive advantage for the first strain.

Mouse models of *H. pylori* lead to evolution of spasmolytic polypeptide-expressing metaplasia (SPEM) as precancerous lesions in the corpus following parietal cell loss. It has been demonstrated that gland colonization, similarly to the antrum, triggers corpus pathology (44). The cellular origin of SPEM is controversial. Early studies revealed a transdifferentiation from chief cells as SPEM origin, while later studies alternatively proposed the origin in regenerative processes initiated by neck progenitor cells (71,72). While the origin of SPEM is unclear and the pathogenesis of SPEM is beyond the scope of this review, it will important to explore whether *H. pylori* triggers corpus pathology by directly interfering with the physiology of parietal cells, as suggested by previous reports (73), or whether similarly to the antrum, the stem cell niche guides epithelial behavior, leading to enhanced proliferation, impaired parietal cell differentiation and development of premalignant lesions.

Outlook

Gastric stem cells are now well defined by various markers and their behavior has been well characterized. Recent evidence revealed that the perturbation of gland homeostasis by infection with *H. pylori* has multiple effects on stem cells, overall resulting in an altered epithelial proliferation and differentiation. Stromal cells show responses to infection and it will be important to further explore the identity of such cells and the regulation of their behavior. In this context, the role of the inflammatory response to *H. pylori* should be taken into consideration, which might act on epithelial cell behaviors, trigger responses of resident stromal cells and facilitate invasion of new stromal cell populations. The study of epithelial behavior upon infection *in vivo*, using the 'tissue microbiology' approach described in this review may provide new insights into the role of virulence factors of *H. pylori*, including well studied factors such as CagA as well as new factors such as ADP heptose. Moreover, it will be important to further dissect the stem cell niche in the corpus in the context of *H. pylori* infection and development of infection-driven premalignant lesions.

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