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Fighting for the right niche: bacterial interactions with gastric epithelial stem cells

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Abbreviations

H. pylori -	Helicobacter pylori
Rspo3 -	R-spondin 3
Lgr –	Leucine-rich repeat-containing G-protein coupled receptor
Fzd –	Frizzled
CagA -	Cytotoxicity associated gene A
PMSS1 -	Pre-Mouse Sydney Strain 1
TFSS -	Type Four Secretion System
IL-1beta -	Interleukin 1 beta
TNF-apha -	Tumor Necrosis Factor -alpha
TLR4 -	Toll Like Receptor 4

1.Introduction

1.1. The stomach

The stomach is part of the gastrointestinal tract, located between the esophagus and the duodenum. It is a luminal organ with the largest diameter within the gastrointestinal tract, and acts as a reservoir to temporarily retain and pre-digest ingested food to allow for efficient absorption of the food contents in the intestine. The digestive function of the stomach is closely linked to its acidic pH, which is also thought to be important for neutralizing pathogens. In addition, the stomach epithelium also secrets enzymes such as pepsinogen that are activated in the lumen to actively break down the ingested food.

1.1.1. Macroscopic Anatomy

The stomach is located in the in the upper part of the abdomen. It is part of the gastrointestinal tract between the esophagus and the duodenum.

The stomach can be divided into sections: the cardia and fundus in the proximal part as well as the two main sections: the body, which is also called corpus and is macroscopically characterized by the presence of mucosal and submucosal folds, the rugae, as well as the more distal part called antrum, where no rugae are visible.

The most proximal landmark is the so called Z-line, which separates the esophagus and the cardia. The Z-line is easy to recognize because two different epithelial types, the stratified eepithelium of the esophagus and the red columnar epithelium of the stomach, meet at this position. In this area, the lower esophagus sphincter is usually located. This structure is important for the regulated transport of the food. Failure of its relaxation can lead to dysphagia, whereas ineffective contraction may increase the risk for acid reflux into the esophagus and induce chemical injury of its epithelium, which is not well equipped to withstand acidic contents.

The distal anatomical landmark is the pylorus, a muscular sphincter structure that regulates further transport of the stomach content into the duodenum.

1.1.2. Microscopic Anatomy

The stomach wall is composed of several distinct tissue layers. The inner layer is the mucosa, which consists of the epithelium, the stromal lamina propria and the lamina muscularis mucosae. The submucosa is a layer of connective tissue and surrounded by a thick muscle layer, the muscularis propria. The muscularis propria itself is assembled of two muscular layers and surrounded by the outer membrane of the stomach, the serosa.

1.2. Gastric Epithelium

1.2.1. Gastric gland cell proliferation and differentiation

The stomach epithelium is composed of a monolayer consisting of various differentiated cell types that are responsible for the stomach functions, such as acid secretion and digestion.

Although the epithelium is composed of a monolayer, it is not flat, but instead invaginated to build anatomical units termed crypts or glands. These glands are monoclonal, meaning that the cells in a gland are thought to derive from one stem cell[1].

The glands in the corpus are characterized by the presence of acid-producing parietal cells[2]. These large cells have a very characteristic structure and their main function is the production of acid. In addition, corpus glands contain zymogenic secretory chief cells that produce pepsinogen and other digestive enzymes[3]. Chief cells are located in the base of corpus glands[3]. The corpus glands have two types of mucous cells, the muc6 producing mucous neck cells and the surface pit cells that produce muc5ac. In addition, tuft cells and neuroendocrine cells are present in the glands. The corpus glands are repopulated by stem cells that are located either in the isthmus of the gland or in the base[1]. While isthmus stem cells are highly proliferative, basal cells act as reserve stem cells[4]. These basal cells in the corpus indeed represent a subpopulation of chief cells that only rarely and very slowly contribute to gland regeneration in the heathy state but do repopulate glands in the context of epithelial injury. The reserve stem cells in the base of the glands express Wnt target genes such as TROY and Lgr5 as well as Mist1[3,4].

The classic antrum glands do not contain any chief and parietal cells, whereas the other cell types are also present in the antrum gland. Muc6 expressing mucous cells are located in the base of antral glands, while Muc5AC positive mucous cells represent the surface pit cells[1].

Although neuroendocrine cells are present in both antrum and corpus, the antrum cells express and secrete different hormones compared to the corpus. Particularly the expression of gastrin is restricted to the antrum[5].

The antrum stem cell compartment is located in the base of the gland. Here Lgr5 is a well-recognized marker of stem cells[6]. Lineage tracing experiments have shown that Lgr5+ cells give rise to entire gastric glands in the antrum but not in the corpus[6]. However, Lgr5+ cells in the antrum appear to be less proliferative as compared to the small intestinal and colonic Lgr5+ cells, and it has been proposed that other subpopulations of stem cells may exist[1].

Stem cells are considered as long-lived cells of the glands[7]. Their division and self-renewal are features that distinguish them from differentiated cells, which are shed into the lumen, and they are assumed to give rise to all the differentiated cells in the gland. In the antrum, a detailed characterization of Lgr5 cell behavior has been performed and a mathematical model has been developed to characterize the behavior of stem cells[8]. It appears that stem cells either give rise the two stem cells or two differentiated cells, indicating a symmetric mode of division. Every gland has a limited number of stem cells that have the capacity to fully regenerate the gland[8]. The probability of each of the stem cells within a gland to give rise to the full gland is identical, which is described by the term neutral competition[8]. Although the turnover kinetics differ between the antrum and intestine, the principles of stem cell competition are similar across these tissues[9]. The exact turnover dynamics in the corpus has not yet been explored in detail probably due to the more complex anatomy of the corpus gland.

While stem cell behavior in the gastrointestinal tract has been well studied and characterized in the healthy state, it appears that perturbations of epithelial homeostasis may completely change the behavior of the stem cell compartment. This has been shown in the corpus gland, where gland base chief cells do not act as stem cells in healthy conditions, but upon induction

of injury these cells are reprogrammed to de-differentiate and rapidly give rise to entire gastric glands[4].

The concept of stem cell plasticity has also been demonstrated in the intestine, where various cell populations including reserve stem cells and differentiated secretory cells are able to de-differentiate and compensate for the loss of Lgr5+ cells – as well as give rise to new Lgr5+ cells[10-12]. These observations led to the hypothesis that the stem cell function, and expression of stem cell associated genes is not a cell-intrinsic feature but is driven by the cellular microenvironment.

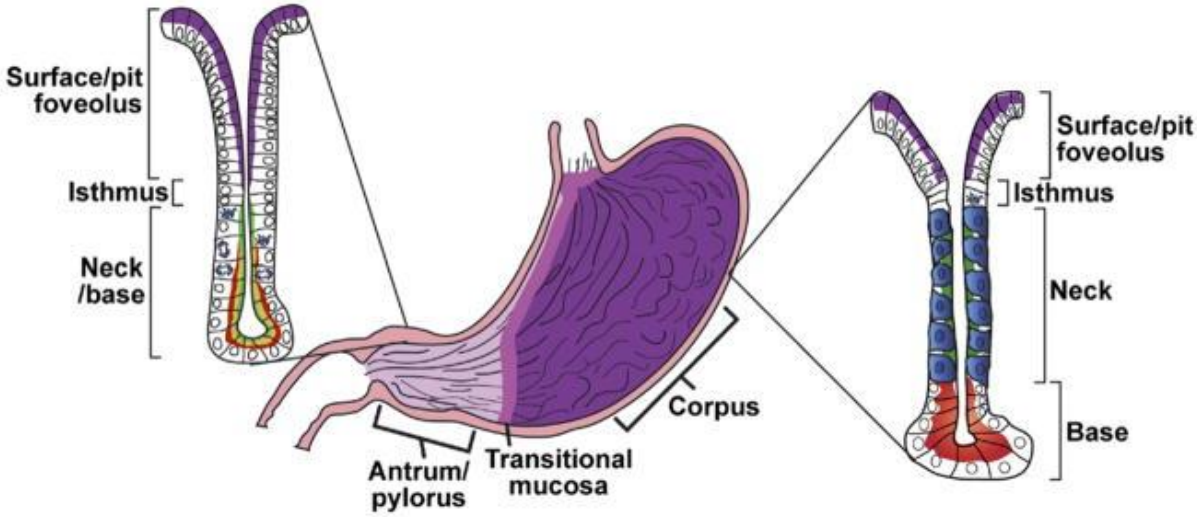

Figure 1

Macroscopic and microscopic structure of the stomach displaying specific cell types of the respective glands. Antral glands consist of stem cells in the base, proliferative cells in the isthmus and surface mucous cells in the pit region, whereas the corpus gland has chief cells in the base, parietal cells in the neck, proliferative cells in the isthmus as well as surface mucous cells in the pit. Reprinted from[1], with permission from Elsevier.

1.2.2. Stem Cell Niche

The microenvironment that surrounds the stem cells is also called the stem cell niche. The niche is built by cells such as myofibroblasts and stromal cells as well as non-cellular material[13]. Stem cell ablation using Lgr5eGFP-DTR mice that have been engineered to express diphtheria toxin receptor and in which diphtheria toxin leads to a rapid apoptosis of Lgr5+ cells, does not have an impact on gut epithelial physiology[14]. It has been shown in the intestine that other cells, such as BMI1+ enteroendocrine cells, de-differentiate and give rise to full crypts, including new Lgr5+ stem cells in the base[14]. This suggested that Lgr5 expression and function of Lgr5+ cells is not restricted to the resident stem cells but instead other cells can re-acquire this function. This so-called stem cell plasticity has been proposed to be regulated by the niche. In parallel, Sato et al have introduced a novel method to culture primary gastrointestinal epithelium ex vivo by applying a 3D organoid technology[15]. The fact that supplementation of specific growth factors to the culture maintains growth of the organoids for long periods of time further substantiated the idea that epithelial stem cell identity and proliferative activity is controlled by niche growth factors. Indeed, small intestinal stem cells were shown to rely on Wnt3 and other growth factors from adjacent Paneth cells[16], and more recently, stromal cells were also shown to be an additional crucial source of Wnt ligands for the intestinal stem cells and epithelial homeostasis[17,18]. While the intestinal stem cell niche has been investigated in some detail, the situation in the stomach is less clear.

1.2.3. Regulation of stem cells by Wnt signaling

Epithelial stem cells in the gastrointestinal tract are known to be proliferative, resulting in a rapid epithelial turnover within days to weeks. The factors that regulate stem cell behavior in the stomach are not well understood. Based on the data derived in the intestine, Wnt signaling is am important regulator of stem cell proliferation. In fact, stem cells are characterized by expression of Wnt target genes, and Lgr5, the most prominent and distinct intestinal stem cell marker, is a Wnt target gene[19]. It has been clearly shown that Wnt ligands are crucial for long-

term culture of epithelial organoids from the stomach [20], but it remained unclear how Wnt signaling is controlled in the stomach *in vivo*.

We have recently extensively reviewed the function of Wnt signaling in the stomach[21], which is summarized below: “Wnt signaling is highly conserved and present in all multicellular organisms. There are 19 known Wnt ligands in humans [22]. These glycoproteins are lipid modified and this process of palmitoylation is thought to be important for the short-range signaling of Wnt ligands, limiting Wnt signaling to specific locations within tissues [22]. Recent data suggest that Wnt ligands reach their receptors through movement in the cell membrane rather than through diffusion. There are two principal signaling routes - canonical and non-canonical Wnt signaling. Canonical Wnt signaling is characterized by translocation of beta-catenin into the nucleus, whereas the term “non-canonical pathway” refers to pathways that do not involve beta-catenin including the planar cell polarity pathway and the non-canonical Wnt/calcium pathway. The type of Wnt ligand and the receptor subtype determine whether canonical or non-canonical pathways are activated [23].

The canonical Wnt pathway is critical in the gastrointestinal tract [23]. Its activity controls stem cell proliferation and de-regulated activation is implicated in gastrointestinal carcinogenesis [3,24-27]. Wnt ligands interact with a receptor called Frizzled and the LRP5/6 receptors, leading to receptor phosphorylation, which in turn inhibits GSK3beta [28]. GSK3beta together with Axin2 and Apc builds the destruction complex. Once Wnt signaling is activated, GSK3beta is inhibited and beta-catenin degradation blocked, enabling its translocation into the nucleus. Beta-catenin then interacts with the transcription factors Tcf/Lef, resulting in the expression of Wnt target genes[29] that involve multiple genes controlling various cellular functions[23]. Wnt signaling is critical for gastrointestinal integrity and turnover, thus inhibition of Wnt signaling can result in a disruption of gut homeostasis.

In the stomach, Wnt signaling is less studied than in the small intestine or colon. During development, the presence of Wnt signaling is lost in the foregut, however in the adult tissue Wnt is present here and one of the aims of the studies presented here was to explore the spatial organization and function of Wnt signaling in the stomach.

In addition to Wnt ligands, other signaling molecules have been shown to modify Wnt signaling. One class of molecules that stabilize Wnt signaling is called Rspo. Four different Rspo homologues have been described. Recently Rspo homologues have been shown to interact with the family of Lgr proteins (Lgr4, Lgr5 as well as Lgr6) [30] to stabilize Wnt signaling. Turnover of the Wnt receptor Fzd is meditated by the E3 ubiquitin ligases RNF43 and ZNRF3, which are responsible for ubiquitination and lysosomal degradation of Fzd [31,32]. This process is induced by Wnt signaling itself, creating a negative feedback loop to limit Wnt signaling. Rspo binds to the extracellular domain of RNF43/ ZNRF3 as well as Lgr4/5/6[31,32]. This leads removal of the ubiquitinases RNF43/ ZNRF3 from the membrane, preventing degradation of Fzd. [31,33].

While R-spondin molecules potentiate Wnt signaling via Fzd, R-spondin and Wnt have been shown to have indispensable, non-redundant roles in intestinal stem cell homeostasis. It has been demonstrated that Wnt-Fzd interaction is critical for stem cell proliferation, whereas the number of Lgr5+ cells is determined by R-spondin [34]. The molecular basis for these differences are not yet fully understood."

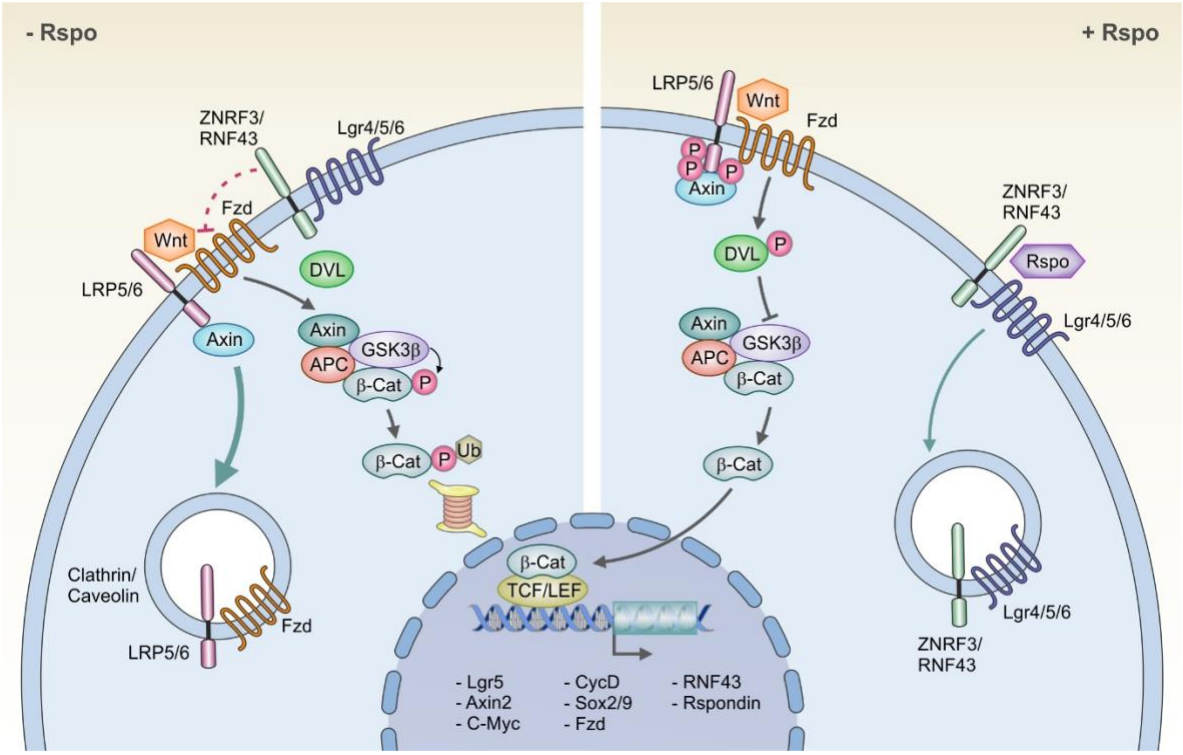

Figure 2 Wnt and Rspo signaling in gastrointestinal epithelial cells. Wnt signaling is mediated upon interaction of Wnt ligands with the receptor Fzd. In the absence of Rspo, Wnt receptors are ubiquitinated by ZNRF3/RNF43 (left), whereas binding of Rspo prevents this process, stabilizing Wnt signaling, Reprinted from [21] under Creative Commons Attribution License which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited (CC BY 4.0)).

1.3. Helicobacter pylori

1.3.1. Helicobacter pylori colonization of the stomach

Helicobacter pylori (H. pylori) are gram negative, spiral shaped bacteria that colonize the stomach of about 50% of the world population[35]. Infection is usually established in early childhood and a transmission from mother to child has been proposed. The human stomach is the only known reservoir for *H. pylori* and once infection is established, it can persist for

decades[36] indicating that it is particularly equipped to survive in the harsh acidic environment. While this might indicate that *H. pylori* are able to live in the environment with a low pH, ir has been shown that instead of surving in the acidic lumen of the stomach, the bacteria have evolved multiple mechanisms to colonize the mucus layer that covers the stomach surface and protects the epithelium itself from the acid. [37]. In addition to the free-swimming bacteria that colonize the mucus, *H. pylori* has been shown to have specific adhesins that allow the bacteria to directly attach to the juctions of epithelial cells [38,39]. The direct interaction with the epithelium has been shown to lead to changes of the cell surface that are induced by *H. pylori* to utilize the epithelium as a site replication [40,41]. The most prominent virulence factor of H. pylori is called CagA and presence of CagA has been linked to gastric pathology. Upon attachment of H. pylori to epithelial cells, CagA is injected into cells using the macromolecular syringe, a type IV secretion system (TFSS) [42]. Injection of CagA leads to multiple changes in epithelial behavior such as loss of polarity and altered assembly of cell junctions [43-45]; activation of signals downstream of receptor tyrosine kinase growth factors [46-49], triggering NF-κB pro-inflammatory responses [49] and activation Wnt signaling [50]. Attachment of H. pylori to epithelial cells and subsequent injection of CagA have been demonstrated to be important for bacterial colonization and persistence [41] as well as for pathological effects of *H. pylori* such as increased epithelial proliferation and tissue hyperplasia. CagA is associated with increased risk of malignant transformation as shown in animal models [51,52], and in epidemiological studies [53].

The direct effects of *H. pylori* on the epithelium have been well established and characterized in cell lines using in vitro experiments. However, the *in vivo* situation is more complex. In contrast to a cell line, the gastric epithelium is organized in glandular gastric units that consist of various differentiated specialized cell types [1]. *H. pylori* have previously been shown to in the mucus layer as well as in direct attachment with surface mucus cells in the gland pits [37]. However, as indicated above, gastric glands undergo constant self-renewal, leading to repopulation of the entire glands every 10 days [8]. Therefore an important question is how the

bacteria are able to induce long-term effects in the epithelium while interacting with terminally differentiated mucous cells that are programmed to die within a short period of time.

1.3.2. H. pylori and stomach disease

While infection of H. pylori in most individuals does not lead to specific symptoms, it is the main risk factor for the development of gastroduodenal ulcers as well as gastric cancer. Approximately 15% of individuals infected with H. pylori have been shown to develop ulcers [54], and another 1% gastric adenocarcinoma, worldwide the third most lethal cancer [55].

One of the most critical questions in the field of *H. pylori* research is the identification of determinants for the outcome of infection.

In the context of gastric cancer, risk factors have been identified that can be divided into host-related, environmental, and bacteria-specific virulence factors. Host genetic factors include genetic variants in innate immune component genes such as IL-1beta, TNF-Alpha as well as TLR4, indicating that a link between immune responses to infection and carcinogenesis could exist. Indeed, overexpression of IL-1beta in a mouse model results in spontaneous development of gastric cancer even without infection. Environmental factors that have been demonstrated to increase the risk for gastric cancer are smoking as well as poor diet, whereas high intake of fruits and vegetables has been suggested to be beneficial and reduce the risk for gastric cancer development. It is well established that infection with *H. pylori* represents the most relevant risk factor for gastric cancer development, Specific virulence factors of H. pylori have been identified to be linked to gastric cancer. Particularly, the Cag pathogenicity island and expression of CagA have been shown to increase the risk[53]. Furthermore a variant of the vacuolating toxin VacA has been linked to carcinogenesis[56].

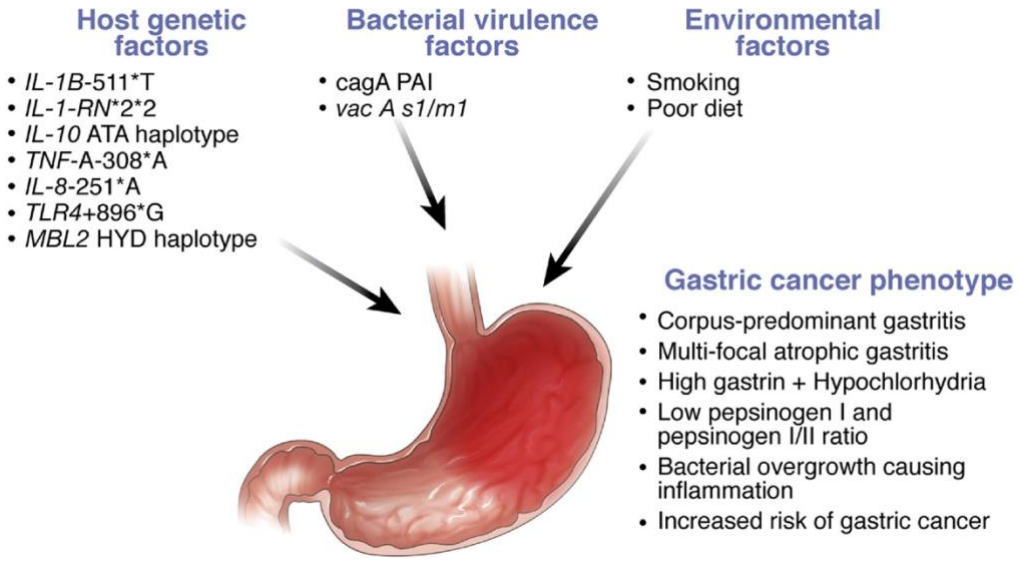

Figure 3 *Summary of factors that contribute to H. pylori-driven carcinogenesis including host-associated factors such as genetic polymorphisms, environmental factors as well as bacterial virulence factors. Reprinted from* [57]*, with permission from Elsevier.*

2. Publications

Publication 1

Sigal M, Rothenberg ME, Logan CY, Lee JY, Honaker RW, Cooper RL, Passarelli B, Camorlinga M, Bouley DM, Alvarez G, Nusse R, Torres J, Amieva MR Helicobacter pylori Activates and Expands Lgr5(+) Stem Cells Through Direct Colonization of the Gastric Glands ***Gastroenterology. 2015*** Jun;148(7):1392-404.e21.

Fighting for the right niche: bacterial interactions with gastric epithelial stem cells

(reproduced from original publication

DOI: https://doi.org/10.1053/j.gastro.2015.02.049):

"BACKGROUND & AIMS: Helicobacter pylori infection is the main risk factor for gastric cancer. We characterized the interactions of H pylori with gastric epithelial progenitor and stem cells in humans and mice and investigated how these interactions contribute to H pylori-induced pathology.

METHODS: We used quantitative confocal microscopy and 3-dimensional reconstruction of entire gastric glands to determine the localizations of H pylori in stomach tissues from humans and infected mice. Using lineage tracing to mark cells derived from leucine-rich repeat-containing G-protein coupled receptor 5-positive (Lgr5(+)) stem cells (Lgr5-eGFP-IRES-CreERT2/Rosa26-TdTomato mice) and in situ hybridization, we analyzed gastric stem cell responses to infection. Isogenic H pylori mutants were used to determine the role of specific virulence factors in stem cell activation and pathology.

RESULTS: H pylori grow as distinct bacterial microcolonies deep in the stomach glands and interact directly with gastric progenitor and stem cells in tissues from mice and humans. These gland-associated bacteria activate stem cells, increasing the number of stem cells, accelerating Lgr5(+) stem cell proliferation, and up-regulating expression of stem cell-related genes. Mutant bacteria with defects in chemotaxis that are able to colonize the stomach

surface but not the antral glands in mice do not activate stem cells. In addition, bacteria that are unable to inject the contact-dependent virulence factor CagA into the epithelium colonized stomach glands in mice, but did not activate stem cells or produce hyperplasia to the same extent as wild-type H pylori.

CONCLUSIONS: H pylori colonize and manipulate the progenitor and stem cell compartments, which alters turnover kinetics and glandular hyperplasia. Bacterial ability to alter the stem cells has important implications for gastrointestinal stem cell biology and H pylori-induced gastric pathology." Abstract from [58].

As demonstrated in the paper above, *H. pylori* appear to actively colonize gastric glands. One important question that has never been addressed is how the bacteria are able to find and locate gastric epithelium. We have hypothesized that bacteria take advantage of a chemotaxis machinery for this purpose and have identified a mechanism of how bacteria identify and swim towards the epithelium:

Publication 2

Huang JY, Sweeney EG, Sigal M, Zhang HC, Remington SJ, Cantrell MA, Kuo CJ, Guillemin K, Amieva MR. Chemodetection and Destruction of Host Urea Allows Helicobacter pylori to Locate the Epithelium. **Cell Host Microbe. 2015** Aug 12;18(2):147-56.

Abstract (reproduced from original publication

(DOI: https://doi.org/10.1016/j.chom.2015.07.002)

"The gastric pathogen Helicobacter pylori interacts intimately with the gastric mucosa to avoid the microbicidal acid in the stomach lumen. The cues *H. pylori* senses to locate and colonize the gastric epithelium have not been well defined. We show that metabolites emanating from human gastric organoids rapidly attract *H. pylori*. This response is largely controlled by the bacterial chemoreceptor TlpB, and the main attractant emanating from epithelia is urea. Our previous structural analyses show that TlpB binds urea with high affinity. Here we demonstrate that this tight binding controls highly sensitive responses, allowing detection of urea concentrations as low as 50 nM. Attraction to urea requires that *H. pylori* urease simultaneously destroys the signal. We propose that *H. pylori* has evolved a sensitive urea chemodetection and destruction system that allows the bacterium to dynamically and locally modify the host environment to locate the epithelium." [59] Together this study demonstrated that *H. pylori* are equipped to actively swim towards stomach epithelial glands.

Our data from paper 1 show that the stem cell compartment can respond to infection. Both, number and proliferation of Lgr5+ cells was increased upon infection of glands with *H. pylori* comparted to uninfected control mice. As mentioned in the introduction, stem cell behavior appears to be driven by the niche and I have hypothesized that Wnt signaling controls stem cells and changes in the niche lead to increased number and proliferation of stem cells upon infection. To address this, I asked how the Wnt signaling in the stem cell compartment is regulated.

Publication 3

Sigal M, Logan CY, Kapalczynska M, Mollenkopf HJ, Berger H, Wiedenmann B, Nusse R, Amieva MR, Meyer TF. Stromal R-spondin orchestrates gastric epithelial stem cells and gland homeostasis. **Nature. 2017** Aug 24;548(7668):451-455.

Abstract (reproduced from original publication (DOI https://doi.org/10.1038/nature23642)

"The constant regeneration of stomach epithelium is driven by long-lived stem cells, but the mechanism that regulates their turnover is not well understood. We have recently found that the gastric pathogen Helicobacter pylori can activate gastric stem cells and increase epithelial turnover, while Wnt signalling is known to be important for stem cell identity and epithelial regeneration in several tissues. Here we find that antral Wnt signalling, marked by the classic Wnt target gene Axin2, is limited to the base and lower isthmus of gastric glands, where the stem cells reside. Axin2 is expressed by Lgr5+ cells, as well as adjacent, highly proliferative Lgr5- cells that are able to repopulate entire glands, including the base, upon depletion of the Lgr5+population. Expression of both Axin2 and Lgr5 requires stroma-derived R-spondin 3 produced by gastric myofibroblasts proximal to the stem cell compartment. Exogenous R-spondin administration expands and accelerates proliferation of Axin2+/Lgr5- but not Lgr5+ cells. Consistent with these observations, *H. pylori* infection increases stromal R-

spondin 3 expression and expands the Axin2+ cell pool to cause hyperproliferation and gland hyperplasia. The ability of stromal niche cells to control and adapt epithelial stem cell dynamics constitutes a sophisticated mechanism that orchestrates epithelial regeneration and maintenance of tissue integrity."[27]

While bacteria can colonize the stomach glands and induce Wnt signaling through an increased expression of Rspo3 in myofibroblasts, we wondered how this response will affect the bacterial colonization. We also noticed that while the overall function of Rspo3 is to induce proliferation in the gland, the basal Lgr5+ cells do not proliferate upon exposure to Rspo3 and addressed how Rspo3 shapes these cells in both heathy state as well as upon infection:

Publication 4:

Sigal M, Reines MdM, Müllerke S, Fischer C, Kapalczynska M, Berger H, Bakker ERM, Mollenkopf H-J, Rotheneberg ME, Wiedenmann B, Sauer S, Meyer TF R-spondin-3 induces secretory, antimicrobial Lgr5+ cells in the stomach **Nature Cell Biology** 21, 812-823 **(**2019).

Abstract (reproduced from original publication DOI https://doi.org/10.1038/s41556-019-0339-9)

"Wnt signalling stimulated by binding of R-spondin (Rspo) to Lgr-family members is crucial for gastrointestinal stem cell renewal. Infection of the stomach with *Helicobacter pylori* stimulates increased secretion of Rspo by myofibroblasts, leading to an increase in proliferation of Wnt-responsive Axin2+Lgr5- stem cells in the isthmus of the gastric gland and finally gastric gland hyperplasia. Basal Lgr5+ cells are also exposed to Rspo3, but their response remains unclear. Here, we demonstrate that—in contrast to its known mitogenic activity—Rspo3 induces differentiation of basal Lgr5+ cells into secretory cells that express and secrete antimicrobial factors, such as intelectin-1, into the lumen. The depletion of Lgr5+ cells or the knockout of *Rspo3* in myofibroblasts leads to hypercolonization of the gastric glands with *H. pylori*, including the stem cell compartment. By contrast, systemic administration or overexpression of *Rspo3* in the stroma clears *H. pylori* from the gastric glands. Thus, the Rspo3–Lgr5 axis simultaneously regulates both antimicrobial defence and mucosal regeneration."[60]

Previous studies have shown that interferon gamma is an important cytokine that mediates antimicrobial defense of the epithelium. However, we also noticed that bacteria are almost never fully cleared from the stomach. Instead some bacteria manage to persist in the stomach despite the strong antimicronbial responses. We therefore asked, how *H. pylori* can manipulate epithelial cells to block secretion of antimicrobial compounds that are secreted by epithelial cells in the context of infection:

Publication 5

Morey P, Pfannkuch L, Pang E, Boccellato F, Sigal M, Imai-Matsushima A, Dyer V, Koch M, Mollenkopf HJ, Schlaermann P, Meyer TF. Helicobacter pylori Depletes Cholesterol in Gastric Glands to Prevent Interferon Gamma Signaling and Escape the Inflammatory Response. **Gastroenterology. 2018** Apr;154(5):1391-1404.e9

Abstract (reproduced from original publication)

DOI: https://doi.org/10.1053/j.gastro.2017.12.008

"BACKGROUND & AIMS: Despite inducing an inflammatory response, Helicobacter pylori can persist in the gastric mucosa for decades. H pylori expression of cholesterol-α-glucosyltransferase (encoded by cgt) is required for gastric colonization and T-cell activation. We investigated how cgt affects gastric epithelial cells and the host immune response.

METHODS: MKN45 gastric epithelial cells, AGS cells, and human primary gastric epithelial cells (obtained from patients undergoing gastrectomy or sleeve resection or gastric antral organoids) were incubated with interferon gamma (IFNG) or interferon beta (IFNB) and exposed to H pylori, including cagPAI and cgt mutant strains. Some cells were incubated with methyl-β-cyclodextrin (to deplete cholesterol from membranes) or myriocin and zaragozic acid to prevent biosynthesis of sphingolipids and cholesterol and analyzed by immunoblot,

immunofluorescence, and reverse transcription quantitative polymerase chain reaction analyses. We compared gene expression patterns among primary human gastric cells, uninfected or infected with H pylori P12 wt or P12Δcgt, using microarray analysis. Mice with disruption of the IFNG receptor 1 (Ifngr1-/- mice) and C57BL6 (control) mice were infected with PMSS1 (wild-type) or PMSS1Δcgt H pylori; gastric tissues were collected and analyzed by reverse transcription quantitative polymerase chain reaction or confocal microscopy.

RESULTS: In primary gastric cells and cell lines, infection with H pylori, but not cgt mutants, blocked IFNG-induced signaling via JAK and STAT. Cells infected with H pylori were depleted of cholesterol, which reduced IFNG signaling by disrupting lipid rafts, leading to reduced phosphorylation (activation) of JAK and STAT1. H pylori infection of cells also blocked signaling by IFNB, interleukin 6 (IL6), and IL22 and reduced activation of genes regulated by these signaling pathways, including cytokines that regulate T-cell function (MIG and IP10) and anti-microbial peptides such as human β-defensin 3 (hBD3). We found that this mechanism allows H pylori to persist in proximity to infected cells while inducing inflammation only in the neighboring, non-infected epithelium. Stomach tissues from mice infected with PMSS1 had increased levels of IFNG, but did not express higher levels of interferon-response genes. Expression of the IFNG-response gene IRF1 was substantially higher in PMSS1Δcgt-infected mice than PMSS1-infected mice. Ifngr1-/- mice were colonized by PMSS1 to a greater extent than control mice.

CONCLUSIONS: H pylori expression of cgt reduces cholesterol levels in infected gastric epithelial cells and thereby blocks IFNG signaling, allowing the bacteria to escape the host inflammatory response. These findings provide insight into the mechanisms by which H pylori might promote gastric carcinogenesis (persisting despite constant inflammation) and ineffectiveness of T-cell-based vaccines against H pylori."[61]

3. Discussion

"War in the gland" – colonization and persistence of H. pylori in the gastric gland

Our data indicate that *H. pylori* are able to locate and swim into gastric glands using their chemotaxis machinery [59] and reach the base of the gland where progenitor and stem cells reside[58]. Although these bacteria are able to colonize the acidic stomach, surprisingly acid itself acts as chemorepellent for *H. pylori*, as shown in recent report[62]. The main chemoattractant for *H. pylori* appears to be urea produced by host cells and TlpB is the essential sensor for urea [59].

These data illustrate that bacterial colonization of the epithelium is a sophisticated, highly regulated process that involves an integration of multiple chemotactic signals, finally enabling *H. pylori* to locate its niche and to establish an infection. Once infection is established, bacteria can persist in the stomach for decades. Most of *H. pylori* are free-swimming in the mucus but some bacteria attach and interact with the epithelium in the gastric glands[58]. It is important to understand whether and how gland colonization and direct interaction of bacteria with epithelial cells could be beneficial for *H. pylori*. Our partially unpublished data using isogenic mutants that colonize the mucus but do not invade into gastric glands indicate that over time bacterial colonization of the stomach is more robust in mice that are infected with wild-type bacteria, suggesting that gland colonization may be beneficial for long-term persistence. While gland colonization could be beneficial simply due to the physical properties of the stomach, as gland-associated bacteria might resist clearance mediated by the peristalsis-driven flow of the stomach content towards the small intestine, direct interaction with epithelial cells has also been shown to have additional benefits for *H. pylori*. One aspect is the extraction of nutrients and micronutrients from the host: it has been shown that CagA that is injected into host cells by attached *H. pylori*, alters epithelial polarity and enables iron extraction from the epithelium[41]. Accordingly, in a model of Mongolian gerbil infection, iron deficiently led to an increased

virulence of *H. pylori*, which was more likely to inject CagA into cells, leading to enhanced cancer development[52]. Another aspect of direct interaction of *H. pylori* with the epithelium is the manipulation of the host responses to infection. It has been demonstrated that antimicrobial proteins such as human beta-defensin 3 secreted by epithelial cells can kill *H. pylori* [63]. Injection of CagA, however, interferes with the self-defense abilities of epithelial cells[63]. In addition, as described here, *H. pylori* extracts cholesterol from host cells to be used as a nutrient source, which in parallel leads to a destruction of lipid rafts required for several cytokine receptors [61]. Therefore it is likely that gland-association has multiple beneficial effects for *H. pylori*, and provides advantages for long-term colonization. On the other hand this interaction appears to be a critical event in the context of epithelial injury and development of premalignant lesions, which are observed specifically in the regions of gland colonization[58].

Once cells colonize the gland, they are also able to reach the base where stem and progenitor cells reside. This appears to be an undesired situation for the host, provoking epithelial responses aimed to clear infection. In fact, a recent report demonstrated that gland base cells have distinct responses to infection that are more pro-inflammatory compared to responses of more differentiated surface epithelial cells[64]. The epithelial responses include an enrichment and activation of gland base stem cells that a) fuel regeneration of the infected and presumably injured gland through an expansion of proliferative Axin2+ stem cells and b) simultaneously induce differentiation of a subpopulation of stem cells in the gland base towards secretory cells that produce antimicrobial compounds to counterbalance bacterial infection[27,60]. These responses result in epithelial pathologies such as gland hyperplasia and probably also metaplasia[58]. Increased epithelial proliferation within the gland per se may explain the increased risk for accumulation of mutations and cancer observed in patients with *H. pylori*, as in fact a correlation between stem cell turnover and accumulation of mutations has been suggested[65]. In addition, while the epithelial responses can restrict bacterial colonization, a complete clearance of stomach colonization is not achieved and some bacteria manage to survive. Thus, the host response to infection is not fully effective and results in chronic mucosal

infection and inflammation. In addition, the remaining bacteria are able to apply their virulence factors, which have been shown to be directly pro-carcinogenic by either inducing DNA damage[66] or altering cell signaling events leading to loss of polarity[43]. The increased proliferative activity within the gland, exposure to the inflammatory environment and direct effects of remaining glandular *H. pylori* on epithelial cell integrity appear to create a "recipe for disaster", leading to development of gastric cancer in a subset of patients. It will be important to further explore the "war in the gland" between the bacteria and the host and to determine factors on both sides that increase the risk for development of disease.

Gastric stem cell control by the niche

We were able to further characterize the stem cell compartment in the stomach and found that it is characterized by expression of Axin2 and Lgr5, which are both Wnt target genes. Wnt signaling has been found to be essential for maintenance of gastric organoids, suggesting that it is essential for stem cell function [64]. In the intestine, Wnt3-secreting Paneth cells are essential for the maintenance of stem cells and therefore they constitute the stem cell niche[16].

In the healthy stomach no Paneth cells are present and therefore it was unclear how Wnt signaling is maintained. We found that Wnt ligand expression does not spatially correlate with the expression of Wnt target genes, instead we identified that the Wnt enhancing molecule Rspo3 dictates the expression pattern of Wnt target genes and determines the identity of the stem cell compartment in the stomach. Consistently, Rspo3 depletion leads to loss of Lgr5 expression and recovery of Lgr5 cells upon depletion is Rspo3-dependent. Recent studies in the small intestine and colon confirm the critical role of Rspo signaling also in the intestine[67,68] and while here Rspo3 depletion can be compensated under physiological conditions, it is indispensable for recovery upon epithelial colonic injury [68].

Interestingly Rspo3 has differential effects on Lgr5+ and Axin2+/Lgr5- cells in the stomach: while Axin2+/Lgr5- cells expand and increase their proliferation, which is also reflected by an

overall increased expression of proliferation markers such as Ki67 in the tissue, gland base Lgr5+ cells do not show increased proliferation but instead differentiate into secretory cells in response to Rspo3. Therefore, Lgr5 lineage tracing is rather inhibited upon Rspo3 treatment. There is so far no mechanistic explanation for the differential responses of Lgr5+ versus Lgr5-negative cells to Rspo3 but we speculate that once a critical level of Wnt signaling is reached, the pro-proliferative effects can be overridden by antiproliferative effects and that cells in the very base of gastric glands, which are exposed to the highest levels of Rspo3, are less proliferative because this point is reached here. We were able to reproduce this phenotype in organoids, showing that very high levels of Rspo can block epithelial proliferation, while levels of Lgr5 and other gland base markers remain high. The cellular mechanism the determines the proliferative state of the cell remains unclear.

We were able to demonstrate that Rspo3-driven differentiation of gland base cells could provide benefits for the host because the secretory cells in the base are able to counterbalance infection with *H. pylori*. Therefore the stem cell compartment is equipped with self-protection mechanisms against bacteria. Similarly, intestinal Paneth cells are Wnt dependent secretory cells in the crypt base of small intestinal crypts and have been shown to produce multiple antimicrobial compounds. Of note, Paneth cell function requires the presence of the microbiota, while germ free mice have dysfunctional Paneth cells[69]. Also in the stomach, the full maturation of antimicrobial cells is *H. pylori* dependent – indicating that the local bacterial flora induces specific antimicrobial defense mechanisms in the epithelium. Because molecules that drive antimicrobial cell maturation also have other critical functions for crypt stem cell turnover and differentiation, bacteria can be viewed as important architects of the gut epithelium. While the effects of bacteria on epithelial gland homeostasis might per se be physiological, as seen in the example of microbiota-induced Paneth cell maturation in the small intestine, they might also bear the risk for development of diseases. Wnt and RSPO signaling ARE critical signals that are required for the response of epithelial cells to infection. However, it is important to note that aberrant activation of Wnt signaling is a critical event in cancinogenensis[21]. In this

context, Rspo molecules have been shown to drive gut carcinogenesis in experimental models and humans[70,71], where Rspo fusions were described in a subset of patients with colorectal cancer[70]. Mutations of RNF43, which ubiquitinates Wnt receptors and which is bound by Rspo to stabilize Wnt signaling is found in about 5-10% of stomach cancer patients. It will be important to study whether increased Rspo3 signaling is directly responsible for cancer development in patients with *H. pylori* and to identify factors that determine whether host responses to infection are beneficial for “keeping the peace” in the mucosa versus leading to a deleterious exacerbation of the “war” created by bacterial infection, resulting in gastrointestinal diseases.

4. Summary

The gram-negative bacteria *Helicobacter pylori* have evolved to colonize and persist in the stomach for decades. Although most patients remain asymptomatic, infection represents the main risk factor for gastric cancer.

I have found that *H. pylori* colonizes not only the mucus layer that covers the epithelium but also the intercellular junctions of specialized epithelial cells deep in gastric glands and demonstrated that this subpopulation of *H. pylori* directly interact with progenitor and stem cells.

The presented studies reveal that *H. pylori* actively sense the epithelium and use urea as chemoattractant to swim towards epithelial cells. Of note, isogenic chemotaxis mutants of *H. pylori* have an impaired ability to colonize the glands, indicating that *H. pylori* possesses sophisticated tools to locate and swim towards the gland to establish a colonization niche.

Once infection is established, gland-associated bacteria, which interact with stem cells, trigger specific epithelial responses. Increased stem cell turnover and proliferation are responsible for gland hyperplasia and metaplasia, which are specifically observed in areas of the stomach with gland-associated bacteria. In addition to the overall increased proliferation in the stem and progenitor cell compartment, a subpopulation of gland-base stem cells that express Lgr5 are differentiating into antimicrobial cells and are able to counterbalance infections by secretion of antimicrobial compounds into the gland lumen such as Intelectin 1. Depletion of these cells leads to an increased colonization and deeper invasion of *H. pylori*. We demonstrate that both stem cell turnover and antimicrobial gland base cell differentiation are driven by the same factor that is expressed in the stromal stem cell niche of the stomach gland. This factor is R-spondin 3 and is secreted by stromal myofibroblasts. R-spondin 3 stabilizes Wnt signaling in gland epithelial cells, leading to an increased expression of Wnt target genes. While overall it acts as a mitogen, gland base cells indeed require Rspo3 to differentiate into

secretory cells and depletion of R-spondin 3 leads to a loss of this cell type and an inability of the glands to counterbalance gland colonization.

Our data demonstrate that *H. pylori* infection alters the epithelial gland homeostasis. R-spondin 3 expression is increased upon infection, leading to both, epithelial regeneration and antimicrobial epithelial responses to infection.

Together we demonstrate how the gland colonization by *H. pylori* creates an ongoing battle between the bacteria and the host. We propose that this battle, which usually continues for decades in infected individuals, can result in dysfunctions of the epithelium and increase the risk for gastric malignancies. Further studies will reveal whether we can use host-derived strategies to win such battles against bacteria in the stomach or at other sites in the body.

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Erklärung

§ 4 Abs. 3 (k) der HabOMed der Charité

Hiermit erkläre ich, dass

- - weder früher noch gleichzeitig ein Habilitationsverfahren durchgeführt oder angemeldet wurde,
- - die vorgelegte Habilitationsschrift ohne fremde Hilfe verfasst, die beschriebenen Ergebnisse selbst gewonnen sowie die verwendeten Hilfsmittel, die Zusammenarbeit mit anderen Wissenschaftlern/Wissenschaftlerinnen und mit technischen Hilfskräften sowie die verwendete Literatur vollständig in der Habilitationsschrift angegeben wurden,
- - mir die geltende Habilitationsordnung bekannt ist.

  Ich erkläre ferner, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis bekannt ist und ich mich zur Einhaltung dieser Satzung verpflichte.

  .... Datum Unterschrift