Pathobiology of *Helicobacter pylori*-induced Gastric Cancer

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Abstract

Colonization of the human stomach by *Helicobacter pylori* and its role in causing gastric cancer is one of the richest examples of complex relationship among human cells, microbes, and their environment. It is also a puzzle of enormous medical importance given the incidence and lethality of gastric cancer worldwide. We review recent findings that have changed how we view these relationships and affected the direction of gastric cancer research. For example, recent data indicate that subtle mismatches between host and microbe genetic traits greatly affect risk of gastric cancer. The ability of *H pylori* and its oncoprotein CagA to reprogram epithelial cells and activate properties of stemness demonstrates the sophisticated relationship among *H pylori* and progenitor cells in the gastric mucosa. The observation that cell-associated *H pylori* can colonize the gastric glands and directly affect precursor and stem cells supports these observations. The ability to mimic these interactions in human gastric organoid cultures as well as animal models will allow investigators to more fully unravel the extent of *H pylori* control on the renewing gastric epithelium. Finally, our realization that external environmental factors, such as dietary components and essential micronutrients, as well as the gastrointestinal microbiota, can change the balance between *H pylori*'s activity as a commensal or a pathogen has provided direction to studies aimed at defining the full carcinogenic potential of this organism.

Infection with *Helicobacter pylori*, a bacterial carcinogen, is the greatest risk factor for gastric cancer—a disease that claims hundreds of thousands of lives per year[1].

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Approximately 75% of the global gastric cancer burden and 5.5% of malignancies worldwide are attributable to *H pylori*-induced inflammation and injury[2], yet the precise mechanisms that regulate cancer development in response to this organism are less well defined. Investigations focused on understanding gastric carcinogenesis have compartmentalized risk factors into categories such as *H pylori* strain variation, host responses and genotypes, and environmental influences. However, recently defined interactions among these categories have increased our understanding of disease risk and progression in individuals with persistent colonization[3]. We review exciting new data from discovery-based approaches and innovative model systems that recapitulate the gastric niche; these have increased our understanding of mechanisms that promote gastric carcinogenesis, within the context of host–microbe interactions.

Interactions Between Microbial and Human Genetic Ancestries

H pylori strains are highly genetically diverse and thrive as freely recombinogenic populations within their cognate human hosts. One technique that has been used to broadly assess and compare the genetic composition of *H pylori* strains is multi-locus sequence typing. Using this technology, Linz *et al.* found that *H pylori* strains segregated into several major clades that reflected the phylogeographic origins of their corresponding human hosts[4]. These findings, in conjunction with previous data establishing a >100,000 year association between *H pylori* and humans, invoke a model of prolonged adaptation in which *H pylori* should become less virulent over time[5–7]. However, this organism remains the strongest known risk factor for gastric cancer, raising the possibility that disrupted co-evolution between *H pylori* and humans may affect pathogenesis.

In many regions of the world, rates of *H pylori* infection and gastric cancer are concordant; in Asia, high prevalence rates of *H pylori* mirror the high prevalence of gastric cancer. However, this association is not universal. For example, the prevalence of *H pylori* infection in Africa is high, but the frequency of gastric cancer is extremely low[8]. In Colombia, the prevalence of *H pylori* is also very high throughout the country (>90% of individuals are infected), but individuals residing in the mountains have high rates of gastric cancer (150 cases/100,000), whereas those on the coast have very low rates (6 cases/100,000)[9]. This disparity in the prevalence of gastric cancer, but not *H pylori*, has provided a unique opportunity to assess the effects of interactions between *H pylori* and human ancestry on gastric carcinogenesis.

Kodaman et al. recently used multi-locus sequence type and single nucleotide polymorphism analyses to assess genetic variations in *H pylori* and humans, respectively, in the Colombian population. Their goal was to determine how their co-evolutionary relationships affect development of intestinal-type gastric cancer[6]. Coastal Colombians comprised an admixture of African, European, and Amerindian genetic variation, whereas the mountain populations were of predominantly Amerindian ancestry, with only a minority of European genetic content. *H pylori* isolates collected from the same subjects contained genetic signatures of multiple ancestries; an ancestral African cluster predominated on the coast whereas a European cluster predominated in the mountains[6], affirming previous results from studies of

these populations[10]. Importantly, specific interactions between microbial and human genetic ancestries clearly predicted risk for intestinal-type gastric cancer.

All persons who fell within the lowest decile of African host ancestry content but who were infected with an *H pylori* strain containing >19.8% African ancestry (a genetic mismatch), had an intestinal-type gastric premalignancy [6]. In terms of quantifiable risk, persons with high (95th percentile) Amerindian ancestry who are infected with *H pylori* strains containing high (95th percentile) African genetic content are predicted to have severe and extensive intestinal metaplasia (Figure 1)[6]. If the same individuals are instead infected with *H pylori* strains of the lowest levels (5th percentile) of African ancestry (genetic match), they are predicted to have only mild gastric atrophy[6].

Gastric atrophy has lower risk of progression to cancer than intestinal metaplasia or dysplasia[11]. A recent study from Sweden predicted that 1/256 persons with normal mucosa, 1/85 persons with non-atrophic gastritis, 1/50 persons with atrophic gastritis, 1/39 persons with intestinal metaplasia, and 1/19 persons with dysplasia will develop gastric cancer within 20 years[12]. Similarly, persons in the lowest levels (5th percentile) of Amerindian ancestry but high African ancestry who are infected with *H pylori* strains of high African genetic content are predicted to have only gastritis (Figure 1)[6]. Interactions between host and pathogen ancestries therefore completely accounted for differences in the severity of gastric injury in these populations; human or *H pylori* genetic variation alone are not sufficient to determine disease susceptibility—a genetic mismatch is also required[7]. This finding implies that the constant genetic adaptation of individual *H pylori* strains to their particular hosts achieved a balance that was disrupted by the acquisition of mismatched strains as diverse human populations began to intermix. An important next step for co-evolutionary studies is to identify differential patterns of concerted selection in paired human and pathogenic loci obtained from other global populations, such as Asia, that differ in terms of cancer risk, human ancestry, and *H pylori* genetic variation.

There is also evidence that more granular interactions between host and pathogen genotypes can alter gastric cancer risk. From the bacterial side, the *cag* pathogenicity island (PAI) is a well-characterized and intensively studied *H pylori* virulence determinant; strains that contain the *cag* PAI increase the risk for distal gastric cancer compared to strains that lack this locus[3]. From the side of human genetics, specific polymorphisms in genes encoding inflammatory cytokines can greatly increase the risk of gastric cancer among *H pylori*-infected persons[13]. El-Omar et al. reported that the odds ratio estimates for distal gastric cancer conferred by polymorphisms in *IL1*, *IL10*, or *TNF* were increased in persons infected with *cag*+ strains compared to the total *H pylori*-infected population[14]. Persons infected with *H pylori* isolates that possess another strain-specific genetic locus, type s1/m1 *vacA* alleles, are more likely to develop hypochlorhydria—a phenotype linked to high-expression alleles of *IL1β* and gastric cancer. The combination of high-risk host genotypes and cancer-associated *vacA* alleles or *cag* genotype similarly markedly increases the risk for gastric cancer, up to 87-fold over baseline[15]. Evaluation of human genetic variation in conjunction with genetic analyses of infecting *H pylori* strains can therefore identify colonized persons at the highest risk for gastric cancer and who may be optimal candidates for antimicrobial intervention.

Virulence Factors

H pylori has evolved the capacity to colonize and persist in one of the harshest environments of the human body, the stomach, which is microbicidal to a large number of species. *H pylori* use their motility, chemotaxis, urease production, and other mechanisms to adapt to the acidic conditions of the lumen and colonize a narrow protected niche near the surface of epithelial cells. In gastric biopsy specimens and in animal models, most of the bacteria are observed to be free-swimming in the mucus gel, and some adhere directly to the surface of the epithelium, but none are found more than 25μm away from the surface of the stomach[16, 17]. The necessity to colonize this narrow space brings the bacteria in close proximity to the epithelium, where they are subject to recognition and regulation by the mucosal immune system. It also allows *H pylori* to deliver bacterial products to the epithelium that modulate its activity and inflammatory responses for its own benefit. The 2 best-studied bacterial factors associated with increased risk of cancer and peptic ulcer are CagA, with its associated T4SS, and particular alleles of the oligomeric toxin VacA. Both of these factors have a number of important effects on the epithelium and the mucosal immune system and can increase the risk of gastric pathology.

CagA is a large protein that varies in size from 120 to 140 kD; it is produced in the cytosol of the bacteria and then threaded through a molecular micro-syinge, the *cag* type IV secretion system (T4SS), across the 2 bacterial membranes and the host cell membrane into the host cell cytosol[18–21]. The *cag* TFSS also translocates peptidoglycan into host cells[22, 23]. The genes that encode CagA and the T4SS are located in a mobile region of the chromosome, the *cag* pathogenicity island (*cag* PAI), that is present in more virulent strains of *H pylori*[24, 25]. Once CagA reaches the host cell cytosol, tyrosines in EPIYA motifs are phosphorylated by host c-SRC and c-ABL kinases;[26] CagA then functions as a eukaryotic signaling hub, creating a scaffold with multiple partners near the host cell membrane (reviewed in [27]).

CagA has multiple effects on epithelial cells. These broadly include stimulating cell proliferation through mitotic signaling pathways such as the PI3 kinase–AKT[28, 29], SHP2, GRB2 and MEK–ERK,[30–32] and β-catenin–WNT pathways. [33–35] CagA also reduces epithelial cell apoptosis by interfering with tumor suppressors such as p53[36, 37] and RUNX3[38]. CagA alters epithelial cell polarity through direct interactions with the polarity protein MAP/microtubule affinity-regulating kinase 2 (MARK2 or PAR1b) [39, 40], and disrupts assembly and signaling through the cell junctions[41, 42]. These direct effects of CagA on epithelial cells could promote cancer development, because transgenic mice and zebrafish engineered to express CagA develop carcinomas even in the absence of inflammation[35, 43]. In addition to its direct effects on epithelial cells, CagA and the T4SS activate inflammatory, NF-κB-dependent signaling[22, 44–46] that leads to recruitment of inflammatory cells, reactive oxygen species-induced damage,[47–49] and wound healing responses, which are all oncogenic. These findings and the epidemiological data linking CagA to gastric cancer risk, have led to the definition of CagA as a bacterial oncoprotein[43].

CagA's effects on epithelial cells are reversible and do not become permanent unless the target cells acquire mutations. CagA therefore induces cellular transformation only in

special circumstances, by inducing accumulation of multiple genetic variants over time. One particularly intriguing emerging concept is that a combination of CagA's signaling functions promotes cell de-differentiation or reprogramming of epithelial cells into more immature, stem-like cells that could be more prone to transformation.

Over the years, our understanding of CagA's function has evolved from its discovery as a bacterial antigenic protein epidemiologically associated with disease to a sophisticated signaling molecule that controls fundamental aspects of epithelial biology. In recent years, several groups have begun to investigate the potential pathogenicity of CagA, along with the cellular context in which CagA exerts its effects in vivo. Researchers are asking question such as what determines where and when CagA is delivered into the host cell? How much CagA is delivered and what cell types are targets? Are there environmental or host factors that affect this process? Importantly, how does CagA benefit the bacterium?

The answers to these questions are not completely understood, but several avenues of investigation are providing insight. For example, CagA delivery to the host cell requires intimate contact between the T4SS and the host-cell membrane. The mechanisms of this interaction are being elucidated, but findings have reinforced the concept that bacterial adhesion to epithelial cells is a multi-step and complex process.

Several bacterial adhesins have been epidemiologically implicated in disease (reviewed in [50]) and affect CagA delivery[51]. The T4SS, per se, has biologic activities independent of CagA. Several component of the TFSS needle, such as CagL, CagY and CagI, for example, bind β1 integrins to facilitate CagA delivery to epithelial cells[52, 53]. Since integrins are baso-lateral protein complexes, inaccessible at the lumenal surface of the epithelium, it not well understood where and when CagA is delivered. The bacterial protease HtrA might be able to disrupt the epithelial junctions to allow the bacteria to reach integrins[54].

Conversely, CagA delivery might not always benefit the microbe, because it also induces inflammatory signals such as production of IL8 and recruitment of neutrophils. To counteract these responses, however, the interaction between bacteria and the epithelium can be modified by genetic molecular switches. For example, expression of CagY, an essential component of the T4SS, can be genetically switched on and off through immune selection, which alters the inflammatory response to promote long-term persistence[55]. The expression of other adhesins by *H pylori*, such as BabA and SabA, which bind glycosylated receptors on the gastric mucosa, are also controlled by molecular switches that finely regulate expression levels in vivo[56, 57]. CagA polymorphisms vary among *H pylori* strains isolated from different human populations; these may influence outcomes of disease. For example, the C-terminal region of CagA contains repeated amino acid motifs (EPIYA) flanked by distinct conserved sequences that vary in their host molecular partners and activity[31]. These tyrosine phosphorylation domains are classified as EPIYA- A, B, C, or D, and differ between Western strains (containing A, B, and C domains) and East Asian strains (containing A, B, and D domains)[31]. The number and types of repeats have been associated with differing risks of carcinogenesis[58]. Recently, it was reported that the EPIYA-B motif binds and activates PI3-kinase and AKT, but many Western strains of *H pylori* carry a single nucleotide polymorphism in this domain that reduces its association with PI3 kinase and also

reduces the risk of gastric cancer[59]. The amount of CagA and its cellular target are also likely to have important roles in pathogenesis, but little is known about the precise location and timing CagA delivery.

VacA, a vacuolating cytotoxin, is asecreted by *H pylori* and can assemble into flower-shaped oligomers when added to various types of host cell membranes[60, 61]. The oligomers function as selective anion channels, so VacA is defined as a pore-forming cytotoxin (reviewed in [62]). VacA pores have a number of effects, including changing the permeability of the plasma membrane[63], causing paracellular leakage in epithelial monolayers[64], affecting endosomal maturation[65], and injuring mitochondria[66]. All *H pylori* strains contain *vacA* genes, but there are multiple alleles that confer various degrees of activities in different assays (reviewed in [67]). The more pathogenic alleles of the toxin cause formation of large, late-endosomal vacuoles inside cells in vitro[68]. VacA also induces apoptosis in many cells, by interfering with mitochondrial function[69]. In addition to its cytotoxic features, VacA acts as a powerful immunosuppressant. It inhibits T-cell development[70] and promotes differentiation of dendritic cells into a tolerogenic phenotype that induces development of regulatory T cells[71].

The simultaneous presence of CagA and VacA in a cell can have important consequences, depending on cellular context and interactions between CagA and VacA[72, 73]. One striking recent example is that VacA reduces the half-life of CagA by stimulating its degradation through the autophagy pathway[74]. Interestingly, in CD44^{+} cells that have some characteristics of cancer stem cells, the half-life of CagA was increased and not affected by the presence of VacA. So, the pathogenic effects of CagA can be exacerbated in the context of immature precursor cells, transformed cells, or stem cells[74].

New Systems for Studying *H pylori*-induced Carcinogenesis

Most studies of carcinogenic mechanisms of *H pylori* have been performed in cancer cell lines, short-term ex vivo primary cell cultures, and infected rodents. However, cell lines that have undergone many passages often contain mutations, and are derived from cancer specimens. In vivo models are expensive and can be time consuming to generate. Isolated ex vivo gastric gland systems are limited by their relatively short life span (e.g., 1 week) and a propensity to become heavily contaminated by fibroblasts[75].

Gastroids effectively bridge in vitro and in vivo models by providing a replentishable culture system that can be readily generated from non-transformed gastric epithelium. Gastroids are 3-dimensional structures with central lumens that contain the major cell types found within gastric glands, and epithelial cells within gastroids are polarized[76]. Further, gastroids can survive for up to 9 months[76].

However, there are limitations with this system. Although gastroids can be used to study direct interactions between *H pylori* and gastric epithelium (reflecting early responses to infection), inflammatory signals from infiltrating immune cells and paracrine signals from stromal or mesenchymal cells also influence pathogenesis—these are not represented in current systems. However, the potential to include inflammatory and stromal cells, such as myofibroblasts, in conjunction with microfluidic technology, in more complex organoid

systems, should permit more detailed investigations into the role of chronic inflammation and injury on *H pylori*-induced gastric carcinogenesis in the future. Several groups have now developed and used gastroid models of *H pylori* infection originating from mouse and human tissues (Figure 2).

Wroblewski et al. used mouse gastroids to investigate the effects of *H pylori* strain-specific virulence factors on aberrant epithelial responses with carcinogenic potential[42]. This group demonstrated that gastroids can develop into a self-organizing, differentiating structure via expansion into single-layered epithelial spheroid structures that consist of mucus cells, parietal cells, G-cells, mucus neck cells, D-cells, and enterochromaffin-like (ECL) cells. Gastroids could be successfully infected with *H pylori* via microinjection (Figure 2), which resulted in mislocalization of occludin at the tight junction, a response identical to what had previously been demonstrated in *H pylori*-infected gastric epithelial cells in vitro and in vivo[77]. Infection of gastroids with *H pylori cagA*[+] wild-type or isogenic mutant strains revealed that increased epithelial cell proliferation and β-catenin nuclear translocation was dependent upon CagA. The use of gastroids also identified a previously unreported mechanism through which *H pylori* may heighten the risk of carcinogenesis: increased snail expression, which was confirmed in human gastric tissue specimens[42]. Overall, these findings indicated that *H pylori* is effectively recognized by gastroids and that this system may be used as both a model for discovery as well as validation. Additionally, this system provides an important opportunity to study gastroids harvested from genetically deficient or transgenic mice in the future.

Other groups have extended these findings by developing human gastroids as a model of pathogenic interactions between *H pylori* and epithelial cells. McCracken et al. reported de novo generation of human gastroids derived from antral tissue via directed differentiation of stem cells[78]. Human gastroids could be successfully infected with *H pylori*, which resulted in an increase in CagA-dependent epithelial cell proliferation[78], confirming previous findings from mouse gastroids. Importantly, intracellular CagA rapidly associated with the c-Met receptor, leading to its phosphorylation[78, 79]. Another group has recently shown that 3-dimensional human gastroids can be converted into a 2-dimensional planar polarized model system[80]. This innovation has added considerable versatility to gastroids as a model for studying interactions between mutant strains of *H pylori* as well as genetically manipulated ex vivo primary cell systems (Figure 2). Bartfeld et al. have generated gastroids from human gastric corpus tissue and shown that gastroids can be directed towards progenitor-like gastric gland lineages or well-differentiated gastric pit lineages via manipulation of WNT and nicotinamide concentrations[81]. Importantly, interactions between *H pylori* and epithelial cells increased when undifferentiated gastric gland organoids were used instead of differentiated gastric pit organoids[81]. These findings indicate that the ability of *H pylori* to interact selectively with stem or progenitor cells may lower the threshold for gastric carcinogenesis.

Effects on Gastric Stem Cells

Several potential models have been developed to investigate how *H pylori* infection and its inflammatory response contribute to the dysregulated growth of long-lived cells and

eventually cancer. These include infection-induced de-differentiation of terminally differentiated epithelial cells into long lived, replicating cells; recruitment of mesenchymal stem cells to gastric glands during tissue damage and repair and subsequent transformation of these exogenous stem cells; and/or direct bacterial effects or inflammatory changes in the resident gastric progenitor and stem cells in the stomach.

Since *H pylori* inhabit the superficial mucus layer overlying the stomach lumen, and adhere to mucus pit cells, it is possible that these terminally differentiated cells are the targets of oncogenic transformation. This would involve de-differentiation into replicating cells and acquisition of oncogenic mutations and cancer stem cell traits. Many studies have provided evidence that CagA has reprogramming potential that could convert somatic epithelial cells into a pluripotent, stem cell-like state, and facilitate the acquisition of mutations. For example, cells expressing CagA or infected with CagA-positive bacteria lose key features of epithelial differentiation and undergo phenotypic and molecular changes associated with stemness and epithelial–mesenchymal transition[42, 82–86]. Also, CagA can aberrantly activate WNT signaling to β-catenin and induce WNT target genes such as the transcription factor CDX1[33–35, 87]. CDX1 can, in turn, induce the expression of several stemness-associated factors, such as SALL4 and KLF5, potentially making cells more pluripotent[88]. Consistent with this observation, *H pylori* infection has been shown to induce ectopic expression of KLF5 in mouse gastric glands[89].

However, surface mucus cells are short lived, with a typical life span of only 1–2 days, so their interactions with *H pylori* or inflammatory factors are limited. *H pylori* causes significant inflammatory responses throughout the depths of the glands as well as hyperplasia during periods of chronic active gastritis, indicated by increased cell division and apoptosis of normal glands[90]. This expands the range of proliferating cells into the upper regions of the glands[90], potentially bringing immature cells into contact with the bacteria.

It is well established that premalignant metaplastic lesions are preceded by multifocal-atrophic gastritis with loss of parietal cells and other differentiated cells[91]. Chronic atrophic gastritis is also accompanied by expansion of immature proliferating cells,[92] which has been reproduced in a mouse model of atrophic gastritis in which parietal cells were genetically deleted[93]. When *H pylori* was introduced into this mouse model of atrophic gastritis, a direct interaction between the bacteria and gastric progenitor cells was observed, and some of the bacteria were internalized by progenitor cells[94, 95]. These studies were among the first to show that adult gastric stem cells could serve as a protective niche where a subpopulation of *H pylori* could reside to avoid clearance[96].

More recently, direct interactions between *H pylori* and gastric precursor cells have been observed, in stomachs mice devoid of atrophy but infected with *H pylori*, and in samples from asymptomatic, infected patients with superficial gastritis. By reconstructing the gastric glands in 3-dimensions using confocal microscopy, these reports describe a subpopulation of *H pylori* that resides deep in the gastric glands. The gland-associated bacteria are distinct from the free-swimming bacteria in the surface mucus in that they grow as microcolonies

that adhere directly to epithelial junctions of gastric precursor cells in the isthmus and in the base of the glands (Figure 3)[97, 98].

In addition, localized inflammatory responses in susceptible niches might also be associated with disease progression. For example, neutrophil infiltration specifically localized to the pit proliferative zone has been described as a characteristic pathologic finding of *H pylori*-induced gastritis[99]. It is therefore plausible that direct interactions between *H pylori* and proliferative progenitor cells or longer-lived stem cells occurs throughout the life of the individual; these interactions may result in direct as well as indirect insults that lead to malignant transformation.

The precise markers that identify progenitor cells and stem cells in the stomach are under intense investigation and not fully understood. The gastric antrum shows similarities with the intestine in that the base of the glands contain stem cells marked by expression of the *Lgr5* gene and controlled through WNT signaling[76]. LGR5 is a G-protein coupled receptor that regulates WNT signaling and marks stem cells in several tissues, including the small intestine, colon, hair follicles, and mammary glands (reviewed in [100]). LGR5$^+$ cells in the base of the antral glands give rise to rapidly dividing progenitor cells in the isthmus of the glands, which, in turn, proliferate and differentiate into all other cell types. Other potential stem cell markers in the antrum include rare cells marked by activity of the villin promoter[101] and cells that express the transcription factor SOX2[102]. The identity of the stem cells in the corpus is less well understood. Similar to the antrum, the proliferative zone of the isthmus region below the gastric pits contains most of the mitotically active progenitor cells, and potential stem cells have been described in this region[103–105].

Loss of parietal cells, which occurs in patients with atrophic gastritis, leads to expansion of a progenitor population in the isthmus marked by CD44 [106]. A subpopulation of mature chief cells can act as progenitor cells and give rise to metaplastic lesions in the corpus[107]. A recent study identified a subset of fully differentiated chief cells present at the base of the corpus glands that express the marker tumor necrosis factor receptor superfamily, member 19 (TNFRSF19 or TROY) and function as more slowly growing stem cells in the corpus[108]. Finally, there is evidence that LRIG1 identifies a distinct population of quiescent stem cells in the stomach, similar to findings in the colon; *H pylori* infection can lead to expansion of this subpopulation in vivo[89, 109].

Stem cells and progenitor cells are important candidate tumor-initiating cells in gastric cancer, and are the source of gastric cancer stem cells. Mouse experiments in which the tumor suppressor gene *APC* was deleted from LGR5$^+$ stem cells revealed rapidly developing adenomas[76]. Likewise, inactivation of the tumor suppressor gene, *Klf4,* in villin-positive gastric progenitor cells increased gastric tumor initiation and progression in mice[110].

Several studies have correlated stem cell numbers or damage and progression of gastric carcinoma. For instance, an immunohistopathology study examined the numbers of LGR5$^+$ stem cells in human gastric mucosa as well as associated DNA damage. It found that patients with gastric cancer and *H pylori* infection had an expanded pool of LGR5$^+$ stem cells in the antrum, and higher amounts of DNA oxidative damage in these stem cells[111].

Several other studies have confirmed that levels of LGR5 mRNA and protein are increased in stomach tissues from patients with gastric cancer[112, 113]. Higher levels of LGR5 may be a marker of poor prognosis for patients with gastric cancer[114].

In addition to transforming potential observed in normal gastric stem cells, in animal models (chimeras with inflammatory conditions), chronic damage can lead to recruitment of bone marrow-derived mesenchymal stem cells that incorporate and transdifferentiate into epithelial cells of the gastric mucosa. In mice with *Helicobacter felis* infection, chronic inflammation, gastric metaplasia, dysplasia, and high-grade intraepithelial neoplasia, inflamed glands were found to contain bone marrow-derived cells. Subsequent neoplastic lesions were derived from bone marrow precursors rather than resident gastric stem cells[115]. Similar findings were recently obtained in a mouse model of spasmolytic polypeptide-expressing metaplasia caused by *H pylori*, in which approximately 25% of metaplastic glands contained cells derived from bone marrow precursors[116].

Regardless of whether or not *H pylori* infection progresses to neoplasia in humans, the bacteria appear to have evolved specialized mechanisms to interact and affect the stem and progenitor cells in the gastric glands. To avoid the gastric lumen, *H pylori* are able to reach the surface of the stomach, adhere to the epithelial cells, and even grow as attached micro-colonies directly on the epithelial junctions deep in the gastric glands[97, 117]. This gland-associated *H pylori* population is more prominent in the isthmus in areas rich in mitotic progenitor cells (Figure 3), and occurs early during colonization of mice and in asymptomatic individuals prior to the development of atrophic gastritis[97, 98]. In mice, LGR5^{+} stem cells can be labeled by expression of green fluorescent protein,[76] and the effects of *H pylori* infection on these cells can be monitored (Figure 4). It was recently reported that, within 2 weeks of infection, and before the onset of chronic gastritis, *H pylori* infection of the gastric glands activated the antral LGR5^{+} stem cells, leading to a doubling of the number of stem cells per gland by 2 months of infection[98]. The activation and expansion of stem cells spatially correlates with glands occupied by gland-associated *H pylori* (Figure 4), and mutant *H pylori* unable to colonize the glands do not activate the stem cells, suggesting that direct interaction between the bacteria and these cells promotes this hyper-proliferation.

Overall, it appears that *H pylori* has evolved the capacity to colonize a specialized niche near precursor and stem cells, and that it manipulates these cells for its benefit[94, 98]. Microscopic localization and interactions between the microbe and the progenitor epithelium could therefore be an important variable in the pathogenesis of gastric cancer. A micro-niche could exist within gastric glandular units that is particularly vulnerable to the oncogenic effects of *H pylori*.

H pylori and Iron

Two important clinical observations highlight the relationship between iron and *H pylori* pathogenesis. One is increasing evidence that *H pylori* infection is associated with iron deficiency anemia (IDA), both in sporadic cases of individuals that present with iron-deficiency anemia refractory to iron supplementation[118] as well as at a population level

related to iron deficiency anemia of childhood[119]. The contribution of *H pylori* to IDA in childhood likely has a significant health impact in resource-poor settings, since IDA affects 30% of the human population and is associated with cognitive and developmental impairment[120]. A second observation is that markers of iron-deficiency are associated with increased risk of gastric cancer[121]. *H pylori* could therefore contribute to low iron states, which can worsen outcomes of *H pylori* infection. Research into the molecular mechanisms of these processes has made some connections between *H pylori* virulence factors and environmental risk factors for cancer.

Iron is an essential molecule for most living organisms, but it is a scarce resource for microbes colonizing the human body, because iron restriction is an ancient innate immune defense against infection. Interstitial and intracellular sources of iron are separated from the bacteria that colonize mucosal surfaces by the epithelial barrier and, within the body, iron is sequestered by high-affinity chelators such as transferrin, hemoglobin, and ferritin[122]. In the inflamed gastric mucosa, neutrophils secrete lactoferrin, which tightly binds free iron at the mucosal surface to starve bacteria[123]. In addition, inflammation induces upregulation of hepcidin, a central regulator of iron metabolism, which activates an iron reduction response to pathogens, by blocking iron uptake in the small intestine[122].

So how does *H pylori* acquire iron in the stomach in the presence of such obstacles, and how is this related to the association between iron deficiency and carcinogenesis? *H pylori* has evolved multiple mechanisms of iron acquisition and each has effects that could contribute to gastric pathology. For example, *H pylori* express several membrane transporters that take up soluble forms of iron such as ferrous ions and ferric citrate molecules[124]. These forms of iron are usually insoluble, but the low pH in the stomach lumen solubilizes dietary ferric iron, and maintains it in solution as a complex with ascorbic acid[125]. Thus, the acidic gastric juice contains a source of soluble iron that can be directly used by *H pylori*. However, *H pylori* may have ready access this iron source, since it cannot survive in the low pH of the stomach lumen, instead remaining close to the epithelial surface where it produces urease to buffer its immediate surroundings. Long-term infection with *H pylori* often leads to a reduction in gastric acid and ascorbic acid secretion which, in turn, reduce the availability of soluble iron for *H pylori* and contribute to IDA[126]. In addition, lactoferrin further reduces the availability of free iron near the mucosa and may also contribute to IDA[123].

The upregulation of hepicidin in response to *H pylori* infection might also reduce iron availability in the host and lead to IDA. For example, *H pylori*-associated iron-deficiency does not respond to oral iron therapy without eradication of the bacteria with antibiotics. Treatment of *H pylori*-infected children who have IDA with oral iron therapy did not reduce serum levels of hepcidin, indicating that eradication of the infection is required to restore normal iron homeostasis[127]. Hepcidin is expressed locally in parietal cells of the gastric glands, and gastric hepcidin increases during *H pylori* infection but normalizes following eradication[128].

Since *H pylori* infection has effects on acid, hepcidin, and lactoferrin that lead to reduction of free iron, it is not surprising that *H pylori* also evolved alternative mechanisms of iron acquisition from the host. Unlike many other commensal and pathogenic bacteria that live at

mucosal surfaces, *H pylori* does not produce siderophores—small molecules that can usurp iron from the host and other microbes. However, in vitro studies with defined media have shown that *H pylori* can actually utilize lactoferrin as a source of iron, but only if it is fully saturated with iron. The same studies showed that *H pylori* can also extract iron from saturated transferrin and from hemoglobin[129]. *H pylori* therefore uses on only dietary iron, but also internal host iron reserves that are sequestered across the epithelial barrier.

How does *H pylori* reach iron sources that are present inside the host across from the epithelium? One possibility is that inflammation leads to epithelial erosions or disruption of barrier function exposing *H pylori* to hemoglobin in red blood cells or serum transferrin. A recent study used elegant in vivo microscopy to show that *H pylori* use chemotaxis to swim to and concentrate at mucosal sites of injury, such as ulcers[130]. Other studies have identified a novel mechanism of iron acquisition across the intact epithelium that links iron uptake to the virulence factors CagA, the *cag* T4SS, and VacA. As noted, a subpopulation of *H pylori* adhere to and grow directly on the epithelial surface forming cell-associated microcolonies deep in the gastric glands[97]. In an in vitro model of infection using polarized epithelia, cell-associated *H pylori* grew on cell surfaces even under conditions of low iron availability that do not support the growth of free-swimming bacteria. This indicates that cell-associated *H pylori* can extract nutrients from or across polarized epithelial cells[117].

CagA and VacA were each shown to be important for the ability to colonize the epithelial surface in conditions of low iron availability[131]. CagA, injected into the epithelium, stimulated the uptake of iron-saturated transferrin into the infected epithelial cells. It also perturbed cell polarity to alter the recycling of transferrin inducing its transcytosis across the epithelium (Figure 5). VacA also contributed to mislocalization of transferrin and its receptor by altering endosomal trafficking. These cellular effects raised the hypothesis that *H pylori* virulence factors have a role for bacterial survival in vivo in conditions of low iron availability. This hypothesis was tested in iron-deficient Mongolian gerbils fed iron-restricted diets before infection with *H pylori*. In an iron-deficient host, wild-type CagA+ *H pylori* were able to thrive, whereas the CagA-isogenic mutants were defective in gastric colonization[131].

These observations were quickly followed by the surprising finding that iron-deficient Mongolian gerbils infected with CagA+ *H pylori* develop more severe inflammation, and accelerated pre-malignant and malignant lesions compared to infected animals fed an iron-replete diet[121]. Furthermore, *H pylori* isolated from iron-deficient animals or maintained *in vitro* in iron-deficient conditions have an increased ability to inject CagA and synthesize higher numbers of T4SS needles upon contact with host cells[121, 132]. The localization of *H pylori* in the mucosa was also different, with greater concentrations of bacteria migrating into the glands in iron-deficient animals. In human populations at risk of gastric cancer, *H pylori* strains isolated from patients with the lowest ferritin levels elicited the highest inflammatory responses when co-cultured with gastric epithelial cells. In summary, recent insights into the role of virulence factors in iron acquisition have provided a conceptual link between *H pylori* virulence, risk of gastric cancer, and iron-deficiency (Figure 5), and suggest that environmental factors such as micronutrient availability can significantly alter the outcome of the infection.

Salt and *H pylori* Virulence

A link between high salt consumption and increased gastric cancer risk has been reported from numerous human studies[133, 134]. Gene expression in several bacterial pathogens, including *H pylori*, can be regulated by salt concentrations[135]. Of interest, transcriptional and proteomic studies have revealed increased expression of *cagA* in response to high-salt conditions[136, 137], but only in certain *H pylori* strains. Based on these findings, Loh et al. sequenced the *cagA* promoter in a population of clinical *H pylori* isolates and identified a unique DNA motif (TAATGA) that was present in either 1 or 2 copies. Salt-induced up-regulation of CagA was detected more commonly in strains containing 2 copies of the TAATGA motif than in strains containing 1 copy,[138] and mutagenesis experiments confirmed that 2 copies of the TAATGA motif were required for salt-induced CagA expression.

The effects of salt on *H pylori* infection and gastric cancer have also been extended in vivo using mouse and gerbil models. One study in mice demonstrated that a high-salt diet increased levels of *H pylori* colonization in the stomach and resulted in increased parietal cell loss[139]. A study using gerbils reported that *H pylori* infection and a high-salt diet could independently induce atrophic gastritis and intestinal metaplasia[140], whereas another investigation revealed that *H pylori* infection and high-salt diets have a synergistic effect on gastric carcinogenesis—but only when gerbils also received a chemical carcinogen[141].

Gaddy et al. recently investigated the effects of a high-salt diet on microbe-induced cancer in gerbils using a unique carcinogenic strain of *H pylori*, 7.13[142]. Gastric adenocarcinoma was detected in a significantly higher proportion of infected animals on a high-salt diet than infected animals on a regular diet[142]. Infected animals fed a high-salt diet also developed more severe gastric inflammation[142]; hypochlorhydria, parietal cell loss, and high levels of gastric mucosal IL1B were detected in the animals that developed cancer. Animals infected with a *cagA* negative isogenic mutant strain and fed a high-salt diet had low levels of gastric inflammation and did not develop hypochlorhydria or gastric cancer. Similarly, a high-salt diet did not cause the development of gastric cancer in uninfected animals[142]. These results confirm that a high-salt diet potentiates the carcinogenic effects of *cagA*+ *H pylori* strains.

Based on these data, there are several potential mechanisms by which dietary elements may augment the development of gastric cancer. The direct effects of dietary constituents on gastric epithelium may damage the mucosa, thereby allowing an increased entry of carcinogens into gastric tissue. Certain dietary components interact with intestinal immune receptors and thereby regulate intestinal immunity[143], and similar responses might occur in the stomach. Diet might influence the composition of the gastric microbiota, or promote expansion of *H pylori* variants with pathogenic properties. Dietary factors could also influence epigenetic alterations—folic acid supplementation protected against the loss of global DNA methylation and reduced the development of gastric inflammation and dysplasia in *Helicobacter*-infected mice[144]. The findings summarized here, however, raise another possibility—namely that dietary constituents directly influence the pathogenic potential of *H pylori* by augmenting the expression and function of cancer-associated microbial virulence determinants.

The Gastrointestinal Microbiota

The role of *H pylori* in gastric carcinogenesis is undisputed. However, other microbes in the gastric or intestinal niche could also affect transformation of gastric epithelial cells (for review, see [145]). Studies demonstrating the effectiveness of anti-*H pylori* regimens on gastric cancer incidence raised this possibility by showing that the cancer-lowering effects of antibiotics may be mediated their effects on non-*H pylori* residents of the gastrointestinal tract[146]. Germ-free hypergastrinemic INS-GAS mice that are mono-colonized with *H pylori* develop pre-malignant lesions at a slower pace than *H pylori*-infected specific pathogen-free INS-GAS mice[147]. This accelerated phenotype was associated with an increase in the proportion of Firmicutes and a decrease in the numbers of Bacteroidetes within the stomach[147]. Rapid progression to gastric neoplasia could be restored in germ-free INS-GAS mice that were pre-infected with a restricted Altered Schaedler's Flora before challenge with *H pylori*[148].

In addition to the role of the microbiota within the stomach, extra-gastric constituents of the microbiome have been shown to affect gastric carcinogenesis in mouse models. Infection of C57BL/6 mice with *H bilis* or *H muridarum* before challenge with *H pylori* significantly reduced the severity of *H pylori*-induced gastric inflammation[149, 150]. In contrast, pre-colonization with a different entero-hepatic *Helicobacter* species, *H hepaticus,* increased *H pylori*-induced gastric injury[150]. These exciting results have provided an important framework for studies that can focus on defining fundamental relationships between *H pylori* and residents of the gastrointestinal microbiome as a means to understand gastric carcinogenesis.

Conclusions

Globally, gastric cancer leads to a high number of cancer-related deaths; increasing our understanding the risk factors for this disease is of utmost importance in identifying the individuals at greatest risk for developing gastric cancer. Infection with *H pylori* is extremely common and in some areas of the world, prevalence rates approach 100%, however, 97%–99% of colonized persons will never develop gastric cancer. The risk of developing gastric cancer is dependent on an opus of interacting components including *H pylori* strain-specific virulence factors, the host genotype, environmental factors such as diet as well as alternations in stem cell populations and the microbiome. Molecular interactions among these factors affect the outcome of long-term colonization of *H pylori*. It is therefore critical that results from mechanistic studies be used to identify persons who are at high risk of developing gastric cancer.

Acknowledgments

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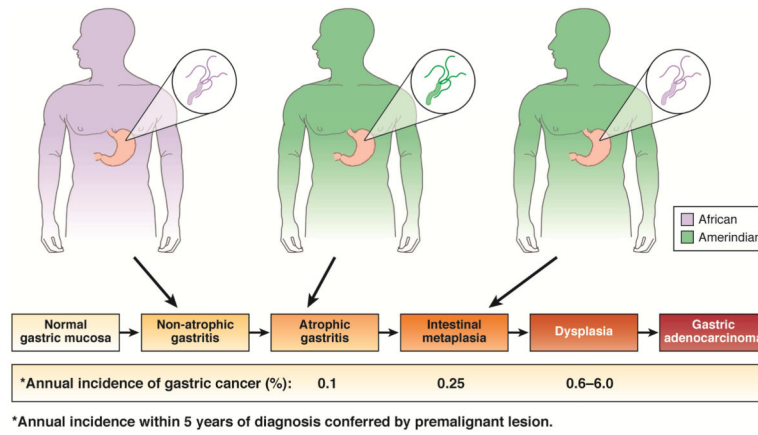
Figure 1.

Interactions between host and *H pylori* genetic ancestries and risk for developing intestinal-type gastric adenocarcinoma in Colombia. Persons with high (95th percentile) Amerindian ancestry who are infected with *H pylori* strains containing high (95th percentile) African genetic content, are predicted to have severe and extensive intestinal metaplasia[6]. If the same individuals are instead infected with *H pylori* strains harboring the lowest levels (5th percentile) of African ancestry (genetic match), the histologic prediction is mild gastric atrophy[6], a lesion with much less risk of progression to cancer[11]. Similarly, persons harboring the lowest levels (5th percentile) of Amerindian ancestry but high African ancestry who are infected with *H pylori* strains of high African genetic content are predicted to have gastritis only[6].
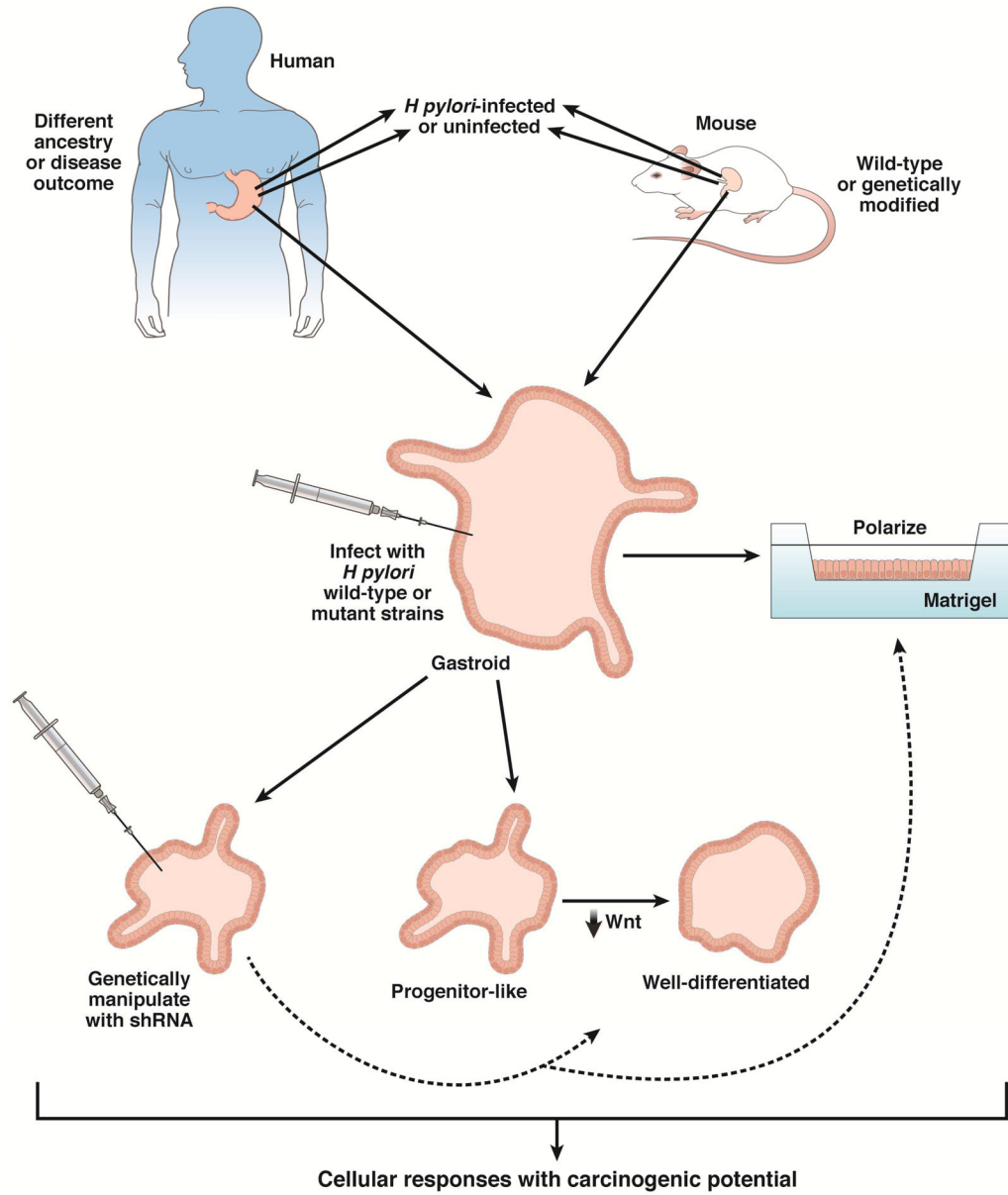
Figure 2.
Translational applications for using mouse or human gastroids to study *H pylori*-induced gastric carcinogenesis.
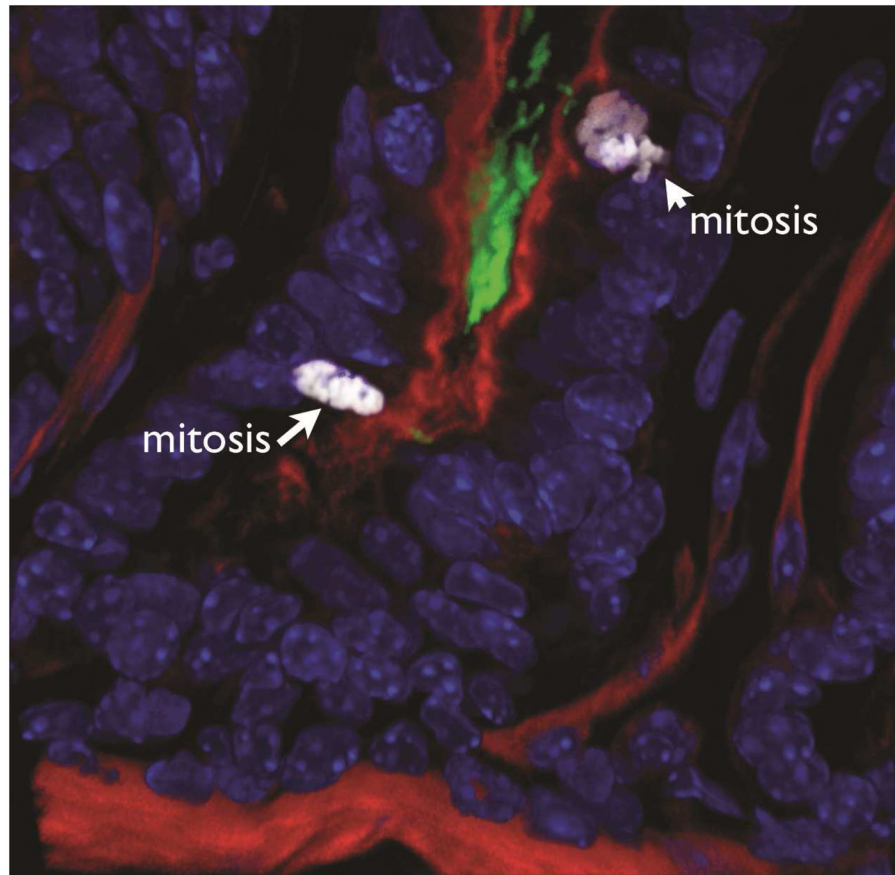
Figure 3.
H pylori colonizes the gland base and interacts with gastric progenitor cells. *H pylori* are labeled green, actin red, and nuclei blue. Cells undergoing mitosis (labeled with ph-Histone) are marked by arrows.
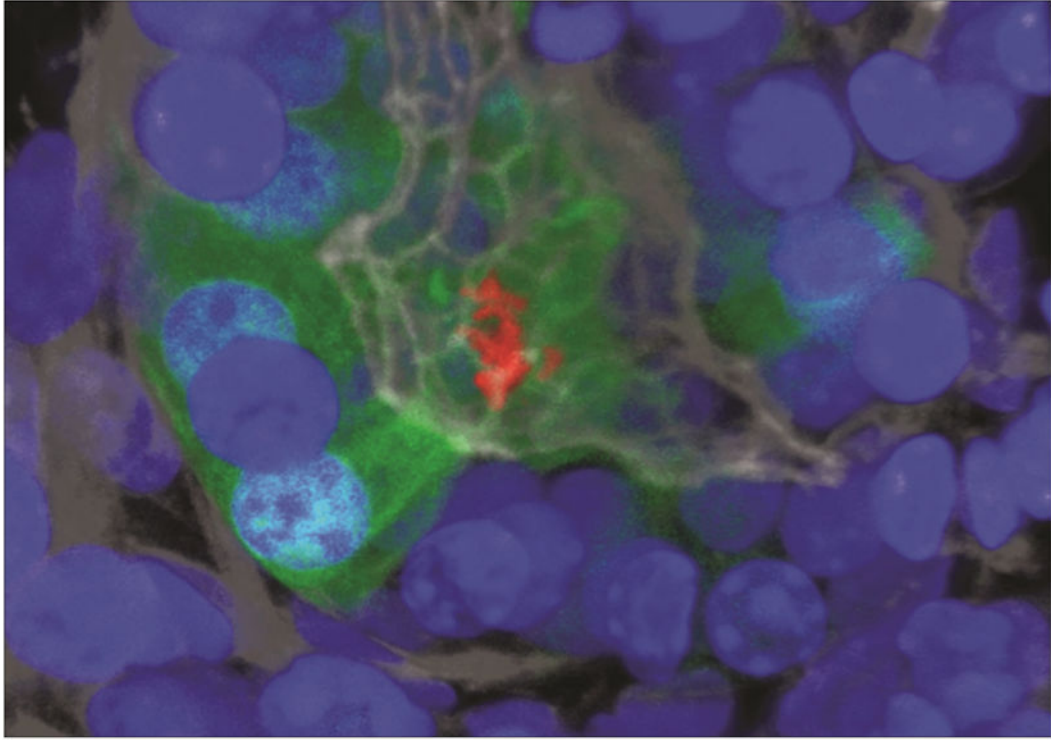
Figure 4.
H pylori micro-colony interacting with LGR5 stem cells at the base of an antral gland. *H pylori* are labeled red, Lgr5$^+$ cells green, and nuclei blue.
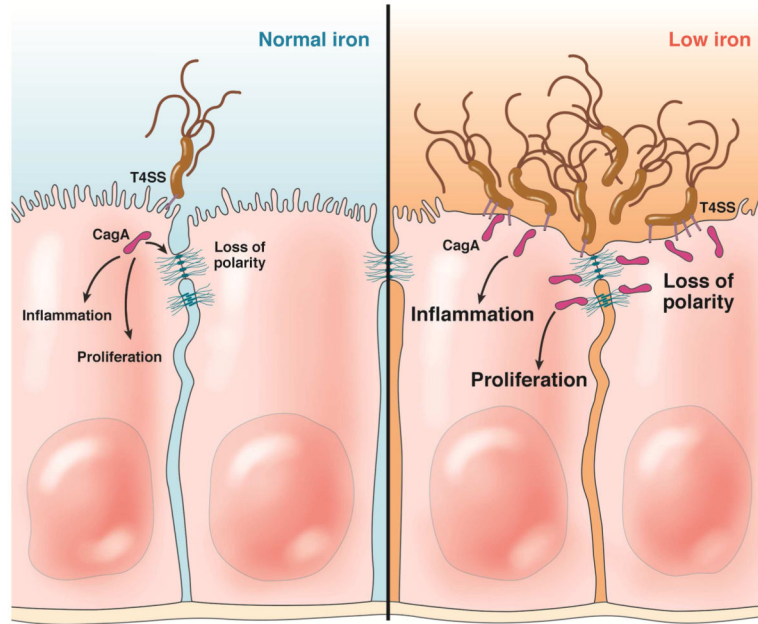
Figure 5.
Gastric epithelial cellular responses with carcinogenic potential that are induced by iron deficiency within the context of *H pylori* infection. The *H pylori cag* T4SS injects CagA into epithelial cells and this results in signaling that induces loss of cell polarity and effects on the epithelial junctions, pro-inflammatory phenotypes, and growth factor-like signaling that results in cellular proliferation. This also induces increased iron uptake and transcytosis. In the right panel, low levels of iron in the host induce increased adhesion and colonization of the glandular epithelium, increased number of T4SS pili, and an augmentation in levels of CagA injection and pathogenic signaling.