DELINEATION OF SIGNALING PATHWAYS INDUCED BY *HELICOBACTER PYLORI* THAT REGULATE HOST CELL SURVIVAL

By

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To my amazing family.

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ORIGINAL PUBLICATIONS

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Franco AT, Johnston E, Krishna U, Yamaoka Y, Israel DA, **Nagy TA**, Wroblewski LE, Piazuelo MB, Correa P, Peek RM Jr. 2008. Regulation of gastric carcinogenesis by *Helicobacter pylori* virulence factors. Cancer Res. **68**: 379-87.

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Wei J, **Nagy TA**, Vilgelm A, Ogden SR, Romero-Gallo J, Piazuelo MB, Correa P, Washington MK, El-Rifai W, Peek RM, Zaika A. Regulation of p53 tumor suppressor by *Helicobacter pylori* in gastric epithelial cells.

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LIST OF ABBREVIATIONS

Abl.....Abelson Murine Leukemia viral oncogene homolog

AJ.....AdherensJunction

ANOVA.....Analysis of Variance

AOM…….....Azoxymethane

AP-1.....Activator Protein 1

APC.....Adenomatosis Polyposis Coli

BabA.....Lewisb Blood Group Antigen Adhesin

BB.....*Brucella* Broth

BCA.....Bicinchoninic Acid

BMDC.....Bone Marrow-Derived Cell

BSA.....Bovine Serum Albumin

BTB/POZ.....Broad Complex, Tramtrak, Bric a Brac/Pox Virus and Zinc Finger

Cag.....Cytotoxin Associated Gene

cDNA.....Complementary DNA

COX.....Cyclooxygenase

Crk.....v-crk Sarcoma Virus CT10 Oncogene Homolog

Csk.....Carboxy-terminal Src Kinase

DMEM.....Dulbecco's Modified Eagle's Medium

DNA.....Deoxyribonucleic Acid

DN-PPAR……..….....Dominant-negative Peroxisome Proliferator-Activated Receptor

DPBS.....Dulbecco's Phosphate Buffered Saline

Dsh……..……..Dishevelled

DTT.....Dithithreitol

E-cadherin.....Epithelial cadherin

ECM.....Extracellular Matrix

EGFR.....Epidermal Growth Factor Receptor

ELISA.....Enzyme-Linked Immunosorbant Assay

EPIYA.....Glutamate-Proline-Isoleucine-Tyrosine-Alanine

ERK.....Extracellular Regulated Kinase

FasL.....Fas Ligand

FBS.....Fetal Bovine Serum

FOXO……..…….Forkhead Box, Sub-group O

GAPDH.....Glyceraldehyde 3-phosphate Dehydrogenase

Grb2.....Growth Factor Receptor-Bound Protein 2

GSK-3β.....Glycogen Synthase Kinase-3β

H&E.....Hematoxylin and Eosin

HB-EGF.....Heparin-binding Epidermal Growth Factor

HBSS.....Hank's Balanced Salt Solution

HPRT……..……..Hypoxanthine Phosphoribosyltransferase 1

IHC.....Immunohistochemical

IKK.....IκB kinase

IL.....Interleukin

iNOS.....Inducible Nitric Oxide Synthase

INS-GAS.....Insulin-gastrin

IκB.....Inhibitor of κB

JAM.....Junctional Adhesion Molecule

JNK.....Jun-N Terminal Kinase

kDa.....Kilodalton

L-Arg.....L-Arginine

Le.....Lewis

LEF-1.....Lymphoid Enhancer Factor-1

LPS.....Lipopolysaccharide

LRP……..Low Density Lipoprotein Receptor-related Protein

mAb.....Monoclonal Antibody

MALDI-TOF.....Matrix-Assisted Laser Desorption Ionization, Time-of-Flight

MALT.....Mucosa Associated Lymphoid Tissue

MAPK.....Mitogen Activated Protein Kinase

MDCK.....Madin-Darby Canine Kidney

MEK.....MAP ERK Kinase

Min.....Multiple Intestinal Neoplasia

MKK…….....MAPK kinase

MKKK.....MKK kinase

MMP.....Matrix Metalloproteinase

MOI…….....Multiplicity of Infection

mRNA.....Messenger RNA

MS.....Mass Spectrometry

mTOR……..……Mammalian Target of Rapamycin

mTORC……..……...Mammalian Rapamycin-insensitive mTOR Complex

Myc……..……...v-myc Myelocytomatosis Viral Oncogene Homolog

NapA.....Neutrophil Activating Protein A

NF-κB.....Nuclear Factor Kappa B

NO.....Nitric Oxide

NOD.....Nucleotide Oligomerization Domain

OipA.....Outer Inflammatory Protein A

OMP….....Outer Membrane Protein

PAGE.....Polyacrylamide Gel Electrophoresis

PAI….....Pathogenicity Island

PBS.....Phosphate Buffered Saline

PBST.....PBS plus Tween

PCR….....Polymerase Chain Reaction

PDK1……..……..Phosphatidyl-dependent Kinase 1

PDGFR……....Platelet-derived Growth Factor Receptor

PGN.....Peptidoglycan

PI3K……..……..Phosphatidylinositol 3-kinase

PIP_2……..…….Phosphatidylinositol-4,5 bisphosphate

PIP_3……..……...Phosphatidylinositol-3,4,5 trisphosphate

PKC.....Protein Kinase C

PPAR.....Peroxisome Proliferator-activated Receptor

PPRE……..…...Peroxisome Proliferator Response Element

PTEN……...…Phosphatase and Tensin Homolog Deleted on Chromosome Ten

PTP.....Protein Tyrosine Phosphatase

PVDF.....Polyvinylidene Difluoride

Rb……Retinoblastoma

RhoA.....Ras Homolog Gene Family, Member A

RNA.....Ribonucleic Acid

RPMI.....Royal Park Memorial Institute

ROS.....Reactive Oxygen Species

RT.....Room Temperature

RTK……Receptor Tyrosine Kinase

RT-PCR.....Reverse Transcriptase Polymerase Chain Reaction

SabA.....Sialic Acid-Binding Adhesin A

SDS.....Sodium Dodecyl Sulfate

SEM.....Standard Error of the Mean

SH2.....Src Homology Domain 2

SHP-2.....SH2-domain Containing-Tyrosine Phosphatase

siRNA.....Small-interfering RNA

Slt.....Soluble Lytic Transglycosylase

SOS……...Son of Sevenless

Src.....v-src Sarcoma Viral Oncogene Homologue

Stsp……Staurosporine

TBS……..Tris Buffered Saline

TBST……TBS plus Tween

TCF.....T cell Factor

TFSS.....Type IV Secretion System

Th.....T helper

TJ.....Tight-Junciton

TLR.....Toll-like Receptor

TNF.....Tumor Necrosis Factor

VacA.....Vacuolating cytotoxin

WT.....Wild-type

ZO-1.....Zona Occludens 1

CHAPTER I

INTRODUCTION

Helicobacter pylori

Helicobacter pylori is a Gram-negative bacterial pathogen that selectively colonizes the human stomach. This organism is a urease-, catalase- and oxidase-positive curved bacillus that possesses 4-5 polar flagella used for motility, and the majority of *H. pylori* strains express additional virulence factors that have evolved to affect host cell signaling pathways (**Figure 1**). Approximately half of the world's population is infected with *H. pylori* and virtually all infected individuals develop coexisting chronic inflammation that persists for the lifetime of the host [221]. Of infected individuals, 10% develop peptic ulcer disease, 1% develop gastric adenocarcinoma, and less than 0.1% develop mucosa associated lymphoid tissue (MALT) lymphoma. Though *H. pylori* infection can be found in all regions of the world, rates of colonization are higher in developing countries than those in developed areas, with most infections being acquired at a young age [86, 87]. These observations are consistent with epidemiological studies suggesting inadequate sanitation practices, low social class and high-density living situations are among the greatest risk factors for *H. pylori* infection [87, 307].

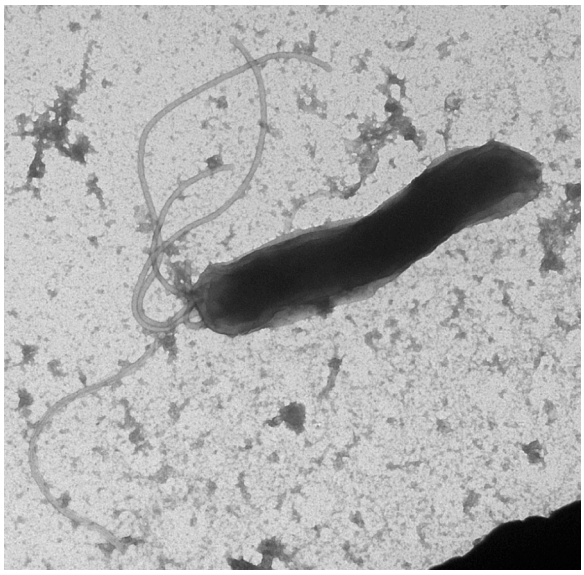

Figure 1: ***Helicobacter pylori.*** Electron micrograph of the Gram-negative pathogen, *Helicobacter pylori*, demonstrating polar flagella used for motility in the mucous gel-layer of the stomach. Micrograph provided by and reprinted with the permission of Aime T. Franco, PhD (Memorial Sloan-Kettering Cancer Center).

Identification of *Helicobacter pylori*

As early as 1875, there were reports of helical bacteria colonizing the human stomach, however, a pure culture of the organism was never obtained [34]. Results from these and similar studies in the early 1900's were forgotten until interest in a stomach-colonizing bacterium was revived with work performed by Robin Warren and Barry Marshall in the 1980's [82, 103]. The pathologist and physician pair successfully visualized bacteria cultured from stomach tissue and argued that most cases of gastritis and ulceration could be attributed to *Helicobacter pylori* (initially named *Campylobacter pyloridis*) infection [176]. Earlier studies by Palmer had demonstrated that in 1,140 gastric biopsies, no spiral bacteria were found, which contributed to the medical community disregarding the initial observations of Warren and Marshall [215]. However, the staining technique used by Palmer was not effective for visualizing *H. pylori*, and Marshall pursued a definitive experiment testing the ability of *H. pylori* to fulfill Koch's postulates by drinking a broth culture of *H. pylori,* documenting the resulting gastritis by serial endoscopies, and finally eradicating the infection with antibiotic treatment [174]. Follow-up studies by Warren and Marshall, as well as Rauws and Tytgat, demonstrated that antibiotic treatment could indeed clear infection, leading to duodenal ulcer healing [175, 238]. Robin Warren and Barry Marshall were eventually awarded the Nobel Prize in Medicine in 2005 for their breakthrough findings on the role of *H. pylori* in gastric disease.

Helicobacter pylori and chronic inflammation

Only a fraction of individuals colonized with *H. pylori* ever develop serious sequelae of infection, yet virtually all patients develop gastritis. Two signature features of *H. pylori* infection are its capacity to persist for decades and the inability of the host to eliminate the organism. Chronic gastritis induced by *H. pylori* is characterized by lymphocyte, plasma cell and macrophage infiltration of the gastric mucosa. Chronic active gastritis may also occur when polymorphonulcear neutrophils are present in the inflammatory infiltrate [117, 176]. Antral-predominant inflammation is typified by hyperchlorhydria and predisposition for duodenal ulceration and conversely, corpus-predominant gastritis is associated with hypochlorhydria, which may lead to gastric ulceration and adenocarcinoma [16]. Duodenal ulceration and gastric adenocarcinoma are two mutually exclusive disease outcomes, thought to be regulated by the degree of inflammation within the gastric mucosa (**Figure 2**) [251].

The adaptive immune response elicited by *H. pylori* is polarized towards the Th-1 cytokine response, which results from recognition of the bacterium by monocytes and macrophages [144, 171, 284]. Specifically, Toll-like receptor 9 (TLR9), an immune pattern recognition receptor of the innate immune system, has been demonstrated to be a key molecule in induction of the Th-1 inflammatory response in *H. pylori*-infected mice by recognizing hypomethylated CpG dinucleotides in *H. pylori* DNA [13].

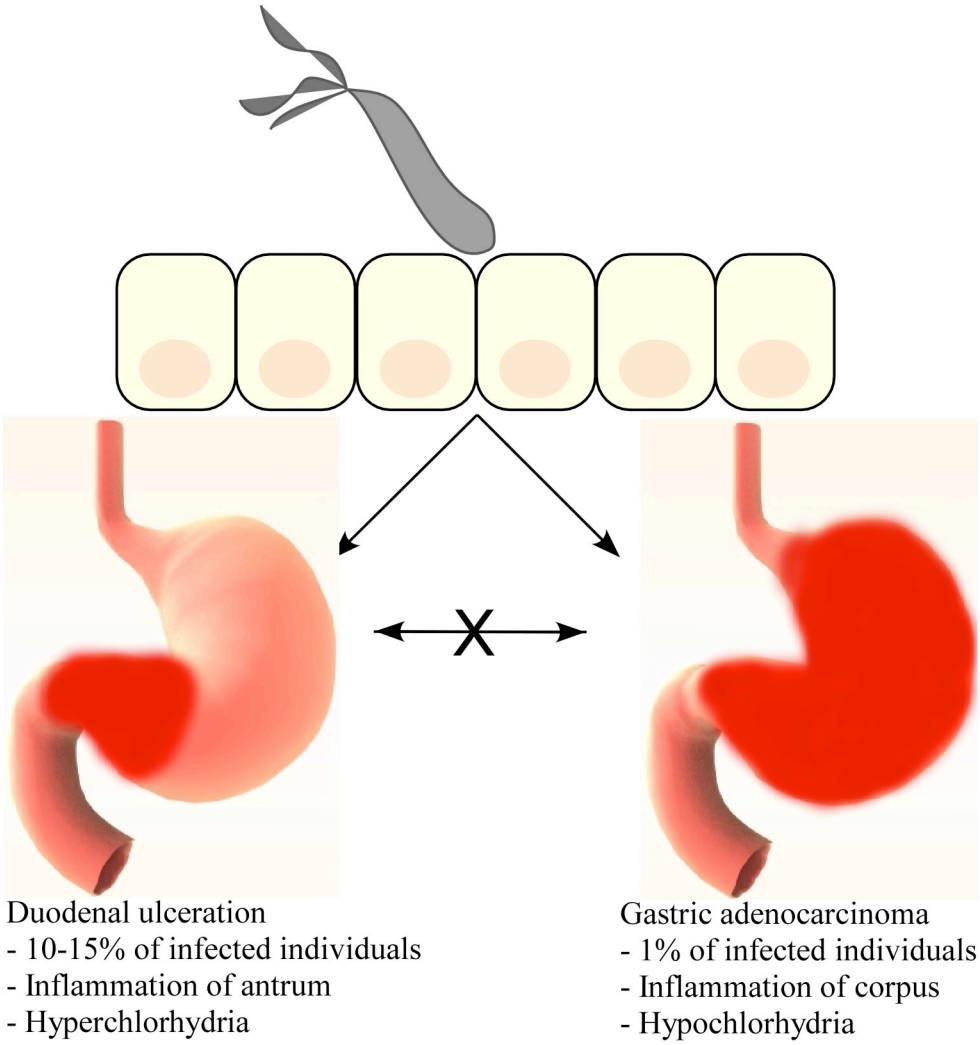

Figure 2. Mutually exclusive disease outcomes associated with *H. pylori* infection. Infection of the gastric mucosa by *H. pylori* results in gastritis in one of two distinct locations, leading to duodenal ulceration or gastric cancer.

H. pylori can also directly stimulate the host immune response via bacterial constituents that are required for its survival in gastric mucosa. The neutrophil activating protein, NapA, is required for *H. pylori* iron-acquisition; however, release of NapA can recruit neutrophils, monocytes and mast cells to sites of infection [193, 350]. Another *H. pylori* virulence factor, Urease, is required for *H. pylori* survival in the acidic gastric environment by converting urea into ammonia and CO_2 [331]. The by-products of Urease are immunogenic, and may also contribute to the immune response against *H. pylori* [116].

Though *H. pylori* elicits a host immune response, this pathogen has developed numerous strategies to aid in its persistence in the gastric mucosa, including evading and limiting the host inflammatory response. Macrophages that encounter *H. pylori* produce nitric oxide (NO), a damaging agent to *H. pylori,* via utilization of a precursor molecule, L-arginine (L-Arg) [106, 115, 336]. However, *H. pylori* produces arginase, encoded by *rocF*, which converts L-Arg to L-ornithine and urea, thereby limiting the amount of L-Arg available to macrophages for production of NO [41, 106, 116, 180, 341]. In addition, *H. pylori* flagella components do not activate TLR-5 and lipopolysaccharide (LPS) of the *H. pylori* membrane is 1000-fold less immunogenic in TLR-4 activation than *Escherichia coli* LPS [111, 194]. Together, these mechanisms contribute to the ability of *H. pylori* to persist in the gastric niche although several of its own constituents can elicit an inflammatory response.

Many tumors arise in the setting of chronic inflammation and the inflammatory response to *H. pylori* may promote carcinogenesis [63]. Hyperproliferation induced by inflammation may lead to an increased risk of mutagenesis, and the production of NO in response to *H. pylori* can also damage host DNA [25, 91, 207, 300]. In addition, there are several microorganisms, such as the Human Papilloma virus, Hepatitis B and C viruses and Epstein-Bar virus that persistently infect humans and induce an inflammatory response, leading to an increased cancer risk [126, 195]. Taken together, these studies have led to the examination of the role of chronic inflammation induced by *H. pylori* in the development of gastric cancer.

The role of *Helicobacter pylori* colonization in the genesis of gastric adenocarcinoma

Gastric adenocarcinoma is the second leading cause of cancer-related deaths worldwide [221]. Because gastric cancer typically invades the muscularis propria prior to diagnosis, mutations frequently occur in metastasis-associated genes (E-cadherin, CD44), and surgery and chemotherapy have minor effects on prognosis, the 5-year survival rate in the United States is less than 15% [59, 285]. Two histologically distinct variants of gastric adenocarcinoma have been described: diffuse-type gastric cancer, which consists of individually infiltrating neoplastic cells that do not form glandular structures, and intestinal-type adenocarcinoma, which progresses through a series of well-defined histological steps defined by Dr. Pelayo Correa in 1975. Intestinal-type adenocarcinoma is initiated by the transition from normal mucosa to chronic superficial gastritis; this is followed by atrophic gastritis and intestinal metaplasia, finally leading to dysplasia and

adenocarcinoma (**Figure 3**) [58, 283]. This form of gastric cancer commonly affects men more than women (male: female 2.1:1) and occurs most predominantly in older patients (50.4 years for men and 47.7 years for women) [60, 124].

One biological consequence of long-term colonization by this pathogen is an increased risk of developing peptic ulcer disease, atrophic gastritis, intestinal metaplasia and gastric adenocarcinoma [28, 58, 93, 122, 132, 143, 148, 154, 186, 203, 219, 231, 279, 282, 315, 330]. Based upon these data, the World Health Organization has classified *H pylori* as a class I carcinogen for gastric cancer and approximately 1% of infected individuals will develop this disease. Eradication of *H. pylori* significantly decreases the risk of gastric cancer in infected individuals without pre-malignant lesions and a randomized prospective study demonstrated eradication significantly reduces the presence of pre-malignant lesions, providing additional evidence that this organism has an effect on early stages of gastric carcinogenesis [181, 337]. Furthermore, our lab and others have shown that eradication of *H. pylori* in experimental Mongolian gerbil infection models results in significant reduction in gastric cancer development [205, 256]. Taken together, these studies further support a role for *H. pylori* in the development of gastric cancer and indicate that anti-*Helicobacter* therapy may be an effective means of gastric cancer prevention.

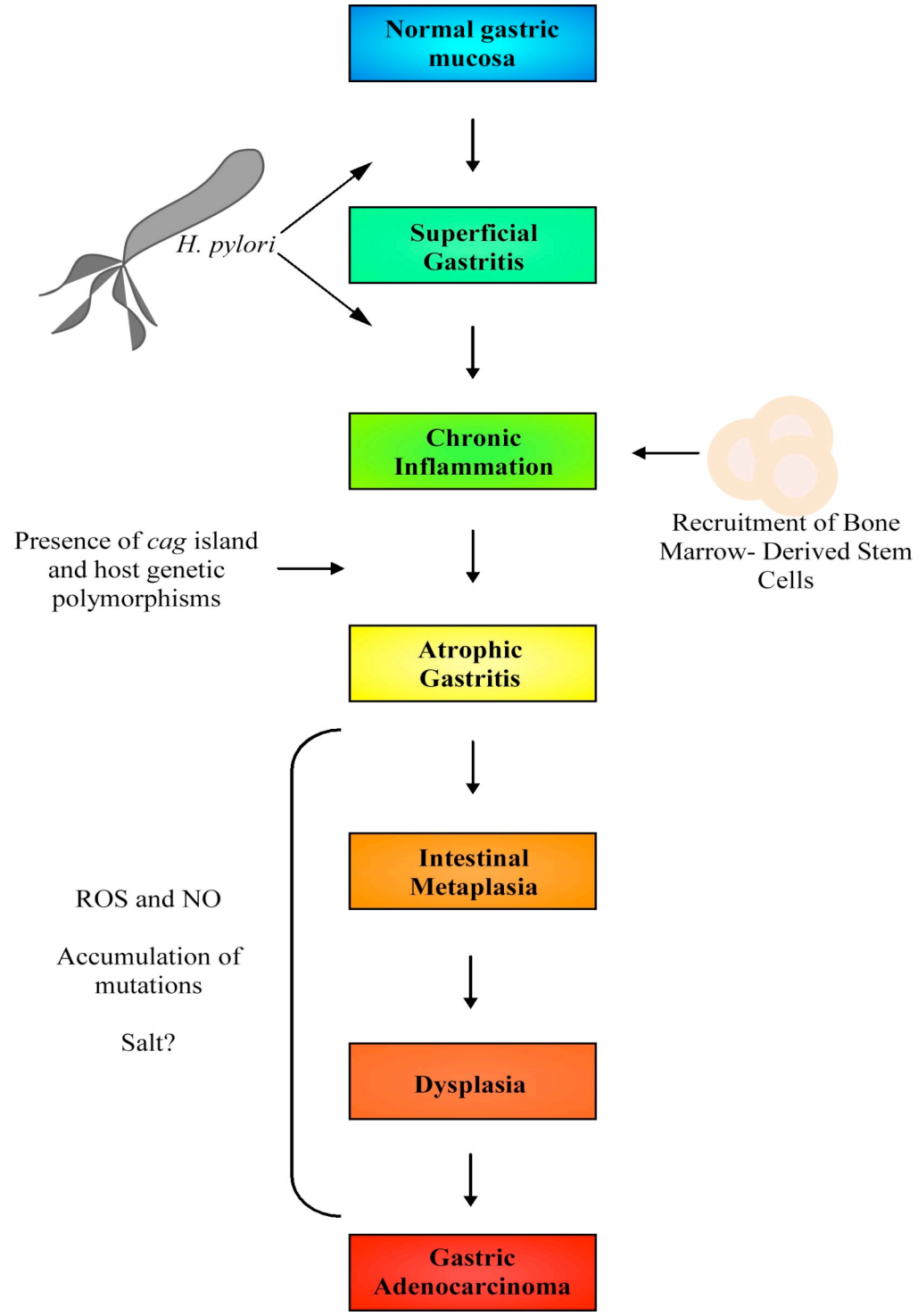

Figure 3. Progression of gastric cancer. The presence of *H. pylori* virulence factors and host genetics influence disease progression. Gastritis occurs within weeks of infection while pre-malignant lesions arise after many years. Adapted from Peek & Blaser and Fox & Wang [95, 221].

Like organs, tumors contain multiple cell types at various stages of differentiation, with their own capacity to propagate. Because of this, it has been hypothesized that tumors possess a stem cell population from which all tumor cells arise-termed the "cancer stem cell" [242, 271]. Prospective cancer stem cells have been identified from tumors of different organs, all having characteristics of peripheral stem cells [6, 57, 90, 134, 261, 280, 281, 325]. Peripheral stem cells have the ability to proliferate under a broad range of conditions and bypass apoptotic stimuli, which may lead to the accumulation of mutations and a predisposition for transformation. The gastric stem cell niche remains elusive and is only implied as the area of highest density containing BrdU-positive cells. It is thought that chronic inflammation induced by carcinogens (such as *H. pylori*) in the stomach and other organs leads to atrophy and specialized cell loss in this niche [235]. The removal of prospective endogenous stem cells has led to speculation another cell type may serve as the cancer stem cell, specifically, a bone-marrow derived cell (BMDC).

Inflammation, chronic injury and atrophy are common characteristics for tissues with increased cancer risk and with this, BMDCs are increasingly found in the peripheral stem cell niche. Persistent tissue injury and exposure to high levels of environmental damage, likely drives transformation of BMDCs. Houghton *et al.* demonstrated that in mice transplanted with genetically- or fluorescently-labeled bone marrow and infected with *Helicobacter felis,* BMDCs repopulated the gastric mucosa and developed into cancer over time [131]. These and similar experiments have helped shape a mechanistic model for the development of gastric cancer in which, chronic inflammation leads to injury and

over time, depletes endogenous gastric stem cell populations (**Figure 3**). These events in turn lead to the recruitment and engraftment of BMDCs, which functionally replace gastric stem cells. As inflammation and injury persist, such as with *Helicobacter* infection for the lifetime of the host, BMDCs are exposed to DNA-damaging free radicals and other mutagens that cause failure to regulate proper growth programs, thus promoting the progression of metaplasia and dysplasia [95]. However, the role of BMDCs in human gastric cancer remains to be elucidated.

Though virtually all individuals infected by *H. pylori* develop chronic inflammation, only a small percentage of colonized individuals ever develop neoplasia, suggesting that enhanced cancer risk involves strain-specific bacterial factors and/or inflammatory responses governed by host genetic diversity, which ultimately determine the interactions between pathogen and host [85, 168]. Several reports also indicate that *H. pylori* infection is inversely related to the prevalence of Barrett's esophagus, esophageal adenocarcinoma, and other diseases such as hay fever, asthma and eczema [37, 48, 49, 51, 75, 166, 316, 321]. These observations underscore the importance of delineation of mechanisms related to microbial-host interactions that will help improve our understanding of *H. pylori*-induced carcinogenesis. Such results would permit physicians to more accurately diagnose and utilize eradication strategies targeted at patients with high-risk for neoplastic transformation.

The *Helicobacter pylori cag* pathogenicity island

H. pylori strains from different individuals are exceedingly genetically diverse due to genomic rearrangements, point mutations, gene insertions and/or deletions [9, 114, 260, 309]. Genetically unique variants of a single strain are present simultaneously within an individual human host, and the genetic composition of these populations can change over time [138]. The identification of bacterial factors associated with disease outcomes has been hindered because of this level of genetic diversity; however, loci have been identified that augment the risk for the development of gastric cancer. These *H. pylori* constituents have the capacity to interact with host molecules and induce epithelial responses with carcinogenic potential.

The *cag* pathogenicity island (*cag* PAI), a 40kB locus, is a well-characterized *H. pylori* virulence determinant that is present in approximately 60% of Western strains [3, 9, 44, 309]. Although all *H. pylori* strains induce gastritis, strains that harbor the *cag* PAI (cag^+) augment the risk for severe gastritis, atrophic gastritis, and distal gastric cancer compared to strains that lack the *cag* island (cag^-) [35, 65, 67, 68, 157, 218, 224, 225, 237, 257, 275, 310, 322]. Studies have shown that *H. pylori* cag^- strains are found predominately in the mucus gel layer while cag^+ strains are found adjacent to and adherent to gastric epithelial cells, demonstrating that the *cag* genotype influences the topography of colonization in the stomach [42]. Several *cag* genes encode components of a bacterial type IV secretion apparatus that acts as an injection apparatus to export bacterial proteins into host cells. One of these proteins, CagE, is required for the

formation of the functional type four secretion system and inactivation of this gene product abrogates delivery of proteins into host cells (**Figure 4**). Another component of the secretion system CagL, functions as a specialized bacterial adhesin that binds to and activates integrin $\alpha_5\beta_1$ receptors, triggering the delivery of bacterial molecules into the cytoplasm of host cells [161].

CagA

The terminal gene product of the *cag* island, CagA, is translocated into host cells after bacterial attachment. CagA is a 120-140kDa protein that contains tyrosine phosphorylation motifs (glutamate-proline-isoleucine-tyrosine-alanine, EPIYA) within the carboxy-terminal variable region of the protein [291]. There are at least four different motif regions within CagA, which are termed EPIYA-A, -B, -C, or –D and are distinguished by the amino acid sequence surrounding the EPIYA motif [124]. Most variants of CagA contain Western-type EPIYA-A, –B and –C motifs, which are phosphorylated to a lesser extent than East-Asian –D phosphorylation sites [124]. Thus, the majority of *cag*$^+$ Western strains are CagA A-B-C and East-Asian strains are A-B-D, though the number of EPIYA-C regions may vary between 1-3 repeated copies among different strains [124].

Following its injection into epithelial cells, CagA undergoes tyrosine phosphorylation by members of the Src family of kinases [15, 22, 208, 267, 269, 292]. Phosphorylated-CagA in turn activates a eukaryotic phosphatase (SHP-2) and extracellular signal-regulated kinase 1 and 2 (ERK1/2), leading to cell scattering, robust actin reorganization known as

the “Hummingbird” phenotype, and other morphologic changes that are reminiscent of unrestrained stimulation by growth factors (**Figure 4)** [15, 22, 127, 128, 208, 267, 269, 291, 292, 312]. Phosphorylated CagA interacts with C-terminal Src kinase (Csk), which acts in a negative feedback loop, to downregulate Src signaling. Non-phosphorylated CagA also exerts effects within the cell that contribute to pathogenesis. Translocation, but not phosphorylation, of CagA leads to aberrant activation of β-catenin, disruption of apical-junctional complexes, and a loss of cellular polarity, alterations that play a role in carcinogenesis [10, 23, 99, 197, 258, 298]

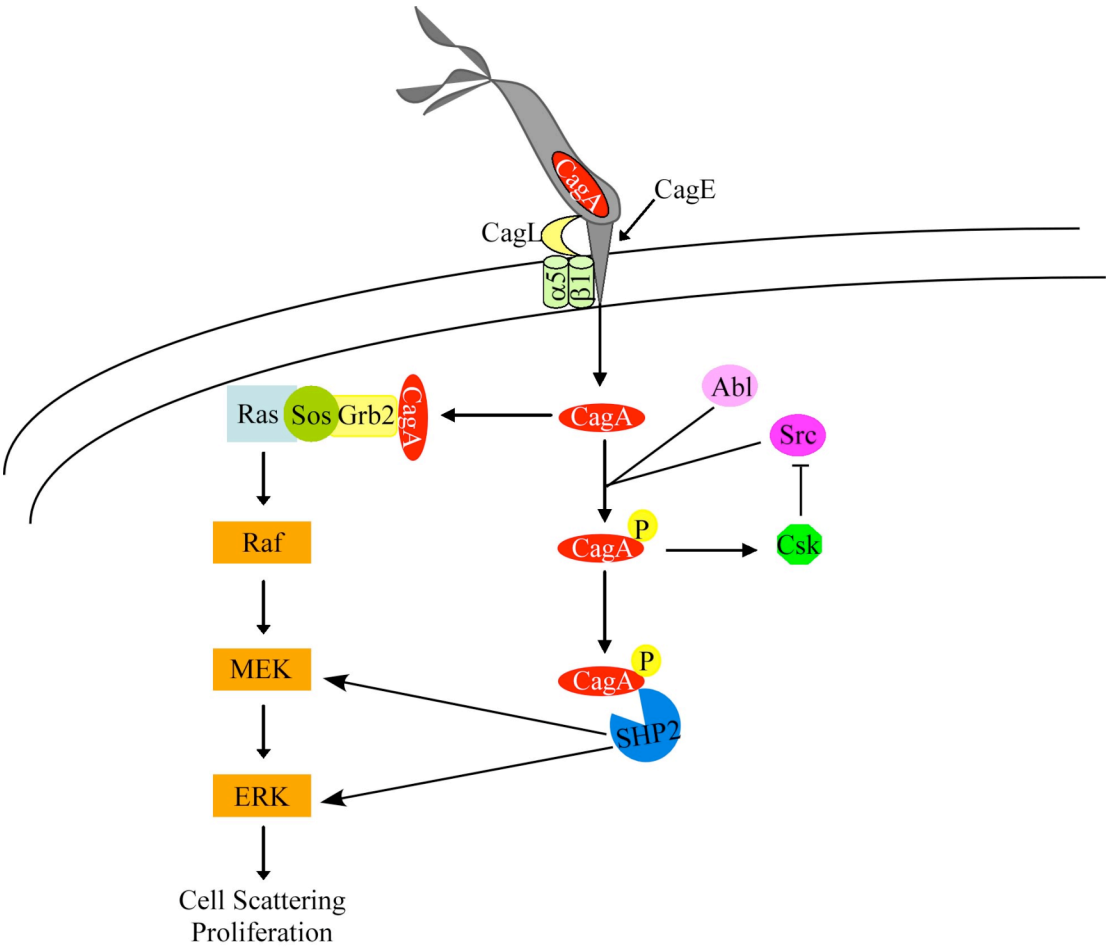

Figure 4. Molecular signaling alterations induced by intracellular delivery of CagA. Translocation of CagA by the secretion system of *H. pylori* leads to activation of host signaling pathways that promote epithelial responses with carcinogenic potential.

Helicobacter pylori peptidoglycan

In addition to CagA, the *cag* secretion system delivers components of *H. pylori* peptidoglycan into host cells where they are recognized by nucleotide-binding oligomerization domain containing 1 (Nod1), an intracytoplasmic pattern-recognition molecule (**Figure 5**) [319]. Nod1 sensing of *H. pylori* peptidoglycan activates NF-κB and regulates expression of the cytokine MIP-2 and β-defensin [39, 319]. The soluble lytic transglycosylase (Slt) enzyme encoded by the *H. pylori* gene *hp0645* functions in anhydromuropeptide turnover and regulates release from peptidoglycan. Mutation of *H. pylori slt* results in an accumulation of G-M-tripeptide in the bacterial peptidoglycan layer (**Figure 5**) [64, 66, 232, 265, 304] and an approximately 40% reduction in the amount of disaccharide tripeptide liberated by the bacteria compared to wild type *H. pylori* [319]. Thus, inactivation of *slt* reduces the amount of peptidoglycan available for translocation by the *cag* secretion system and accordingly co-culture of an isogenic *H. pylori slt*$^-$ mutant with cells expressing exogenous Nod1 attenuates NF-κB activity and IL-8 synthesis when compared to co-culture with wild type *H. pylori* [319].

Another CagA-independent consequence of *cag* island-mediated *H. pylori*-epithelial cell contact is activation of mitogen-activated protein kinase (MAPK) [145, 183, 202]. MAPKs are signal transduction networks that target transcription factors and participate in many cellular functions, including cytokine expression, proliferation, and apoptosis [107, 136, 264]. In mammalian systems, at least five MAPK cascades have been identified including ERK 1/2, p38, and c-Jun N-terminal kinase (JNK). Our laboratory and others have demonstrated that *H. pylori cag*$^+$ strains selectively activate p38, ERK

1/2, and JNK in gastric epithelial cells *in vitro* [40, 61]. *H. pylori* cag^+ strains also transactivate the epidermal growth factor receptor (EGFR) via activation of heparin binding-epidermal growth factor (HB-EGF) [146, 323]. Thus, cag^+ strains augment the risk for serious sequelae of *H. pylori* infection and contact between cag^+ strains and epithelial cells activates signaling pathways that may regulate cellular responses with carcinogenic potential.

Independent cancer-associated loci within the *Helicobacter pylori* genome

An independent *H. pylori* locus linked with gastric cancer is *vacA*, which encodes the secreted bacterial toxin VacA [64, 66, 232, 265, 304]. *In vitro*, VacA induces the formation of intracellular vacuoles and it has been shown to actively suppress T cell activation, which may contribute to the longevity of *H. pylori* colonization [38, 108, 162, 295]. *vacA* is present in virtually all *H. pylori* strains examined; however, the cytotoxin activity varies between strains due to variations in *vacA* gene structure [66, 317]. *H. pylori* strains that possess a type s1/m1 *vacA* allele are associated with an increased risk of gastric cancer compared to those strains that possess s2/m2 alleles [83, 167, 188, 286]. Additionally, *vacA* possesses the i1 or i2 intermediate region allele and Rhead *et al.* demonstrated that among 42 Western strains studied, s1/m1 *vacA* contained the i1 allele and showed high levels of vacuolating activity, while s2/m2 *vacA* alleles were i2 type that were non-vacuolating. This study also demonstrated that the *vacA* i1 allele was a significant marker of gastric cancer-associated strains [248].

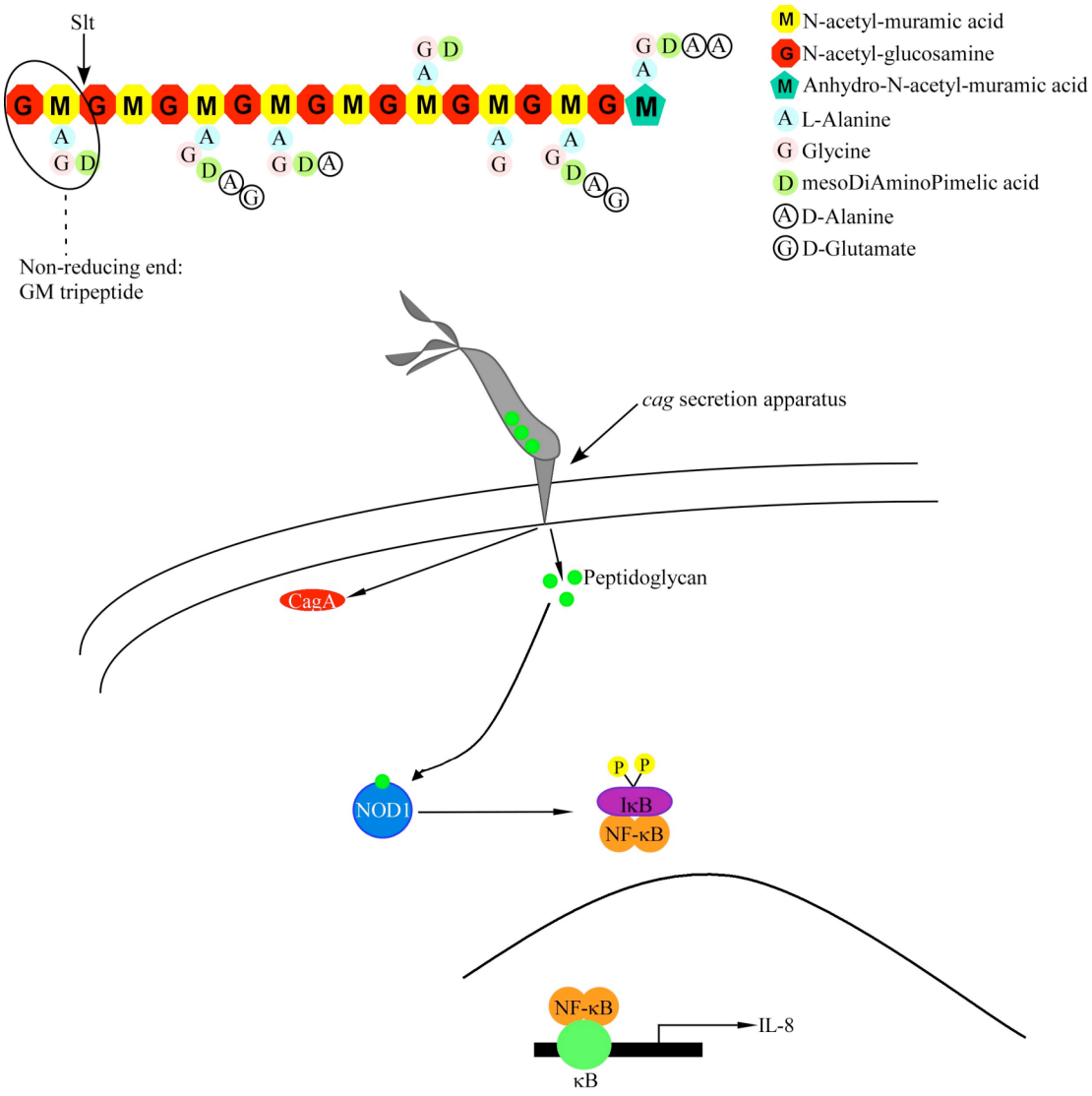

Figure 5. ***H. pylori*** **peptidoglycan stimulates NF-κB.** Upper panel: *H. pylori slt* encodes an enzyme that regulates release of GM tri-peptides during peptidoglycan turnover. Adapted from Chaput *et al.* [47]. Lower panel: Translocation of *H. pylori* peptidoglycan results in the induction of pro-inflammatory cytokines.

Sequence analysis of the genomes of three *H. pylori* strains, 26695, J99 and AG1, has revealed a large proportion of identified open reading frames that are predicted to encode outer membrane proteins (OMPs) [9, 309]. BabA is an OMP encoded by the strain-specific gene *babA2*, which binds the Lewis^b histo-blood-group antigen on gastric epithelial cells; *babA2*$^+$ *H. pylori* strains are associated with increased risk for gastric cancer [110, 135]. The *H. pylori* adhesion SabA binds the sialyl-Lewis^x antigen, which is an established tumor antigen and marker of gastric dysplasia that is up-regulated by chronic gastric inflammation [170].

Rodent models of infection

Several rodent models have provided valuable insights into the host, bacterial, and environmental factors involved in gastric carcinogenesis [253, 287]. Long-term (>1 year) *H. pylori* infection of Mongolian gerbils can lead to inflammation-induced gastric adenocarcinoma, without the co-administration of known carcinogens and gastric cancer development in this model occurs in the distal stomach, as in humans [130, 211, 329, 352]. Prior to 2005, the development of gastric cancer in gerbils had not been demonstrated outside of Japan or China [130, 211, 329, 352]. Our group has now demonstrated that the gerbil-adapted strain 7.13 can induce adenocarcinoma in 17% of challenged gerbils by 4 weeks and 59% of gerbils by 8 and 16 weeks [99, 100]. Mongolian gerbils however, are outbred with undefined genetic backgrounds, which tend to increase the variability of responses to any stimulus. Moreover, compared with mice,

gerbils are relatively poorly characterized and few gerbil-specific reagents are available for detailed investigation.

The ability to utilize inbred mice with defined genotypes allows for more detailed analysis of host susceptibility to *H. pylori* virulence determinants and pathological consequences. One host determinant that may influence the development of gastric cancer is gastrin. Gastrin stimulates gastric epithelial cell proliferation *in vitro* and transgenic mice that over-express gastrin (INS-GAS) develop gastric cancer spontaneously, but this requires the lifetime of the animal (2 years) [139, 326]. Infection of INS-GAS mice with *Helicobacter pylori* or with the related *Helicobacter* species*, H. felis*, accelerates the time to progression to cancer, suggesting that persistently elevated gastrin levels synergize with *Helicobacter* to augment cancer development [326].

H. pylori-infected gerbils and humans with hypergastrinemia and corpus-predominant gastritis develop parietal cell loss similar to infected INS-GAS mice [158]; however, most adenocarcinomas in gerbil or human tissue occur in the antrum, whereas cancer in INS-GAS mice develops most frequently in the corpus [94, 96, 130, 326, 329]. Thus, human gastric carcinogenesis is associated with features present in both *H. pylori*-infected gerbils and mice, which supports the utility of examing *H. pylori*-induced sequelae in multiple model systems.

Activation of PI3K-AKT in carcinogenesis

The phosphatidylinositol 3- kinase (PI3K) pathway is conserved from yeast to mammals and regulates multiple cellular processes including metabolism, survival, proliferation, apoptosis, and cell migration. Members of the PI3K family are lipid kinases that phosphorylate the 3' –hydroxyl group of phosphatidylinositols and both structure and substrate specificity dictate classification of PI3Ks into one of three groups. Class I PI3Ks phosphorylate phosphatidylinositol- 4,5-bisphosphate (PIP_2) to generate phosphatidylinositol -3,4,5-trisphosphate (PIP_3) lipid second messenger (**Figure 6**), whereas Class II and III use phosphatidylinositol as a substrate. The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) lipid phosphatase directly opposes the activity of PI3K by dephosphorylating PIP_3 to generate PIP_2. A link between the PI3K pathway and cancer was first established when its lipid kinase activity was associated with two viral oncoproteins- the src protein of Rous sarcoma virus and the middle-T protein of polyoma virus. Somatic mutations in human cancer target the catalytic subunit of PI3K, p110α (*PIK3CA*), and PTEN at a high frequency, resulting in increased activity of PI3K. Overexpression of *PIK3CA* most often occurs in prostate, breast, endometrial, and colon cancers, but induced PI3K-AKT signaling is also increased in gastric cancer specimens and phosphorylation levels of the primary PI3K target, AKT, correlate with advanced stages of disease [14, 153]. Class IA PI3Ks transduce signals downstream of oncogenic receptor tyrosine kinases (RTK) and *PIK3CA* is the only PI3K gene identified with common mutations in human cancer.

PI3K becomes activated upon binding of a ligand to its cognate receptor tyrosine kinase, such as epidermal growth factor binding to epidermal growth factor receptor. Src kinases, acting both downstream and upstream of EGFR, can also activate the PI3K signaling cascade. Activation of PI3K and the subsequent generation of lipid second messengers recruits its downstream effector, AKT (also known as PKB) to the cell membrane where it is fully activated by phosphatidylinositol-dependent kinase 1 (PDK 1) phosphorylation at threonine 308 and at serine 473 by the rapamycin-insensitive mTOR complex (mTORC2) (**Figure 6**) [45]. Studies now indicate that ubiquitination of AKT by TRAF6 promotes AKT translocation to the plasma membrane and subsequent activation [344].

AKT mediates the downstream effects of PI3K by phosphorylating multiple targets that regulate diverse cellular functions including growth, proliferation and survival (**Figure 6**). AKT-mediated phosphorylation of the transcription factor FOXO can increase proliferation by preventing FOXO from transcriptional activation of cell-cycle regulatory genes *p27Kip1* and pro-apoptotic genes *FasL* and *Bim.* In addition, AKT-mediated phosphorylation inhibits the pro-apoptotic activity of the protein BAD, and glycogen synthase kinase- 3β (GSK-3β), which modulates glucose metabolism, cell-cycle regulatory proteins, and β-catenin. MDM2 promotes degradation of the tumor-suppressor p53 and phosphorylation of MDM2 by AKT promotes this function. In addition, AKT-dependent phosphorylation of NFκB enhances activity, thereby promoting the anti-apoptotic properties of NFκB. In the absence of survival stimuli, Bcl-2 homology domain 3 (BH3)-only proteins interact with and inhibit pro-survival factors at

the mitochondrial membrane, resulting in release of cytochrome c and subsequent death protease activation; however, AKT-dependent phosphorylation of BH3-only proteins disrupts their binding to the pro-survival proteins, thus inhibiting BH3-only-dependent cell death [72, 77]. Additionally, inhibitory phosphorylation of pro-caspase 9, a critical inititator/effector of apoptosis [43], reduces rates of apoptosis, thereby promoting cell survival and enhancing susceptibility of cells to malignant degeneration. Similarly, *H. pylori* infection increases cell proliferation and attenuates apoptosis in humans and rodent models of infection but the mechanisms underlying these findings are not clearly defined [160, 169, 226, 277]. Recently, one study determined that MEK/ERK activation in response to *H. pylori* results in increased Mcl-1 levels (a BH3-only protein), leading to epithelial cell resistance to *H. pylori*-induced apoptosis [190].

In addition to cancer-related processes such as cell-cycle progression and survival that are regulated by AKT activation downstream of PI3K, the lipid second messengers generated by PI3K can regulate cell motility and invasion through Rac and Cdc42 [45]. Cellular migration plays an important role in invasion and metastatic growth of cancers. Although *H. pylori* can increase gastric epithelial cell migration, the mechanisms required for this response are not clearly defined [5, 192, 294]. Recently, it was reported that EGFR transactivation increases intestinal epithelial cell motility in a PI3K- and Src-dependent manner through Rac1 activation [80], so it is possible gastric cell migration in response to *H. pylori* occurs in a similar fashion.

PI3K signaling plays a crucial role in normal developmental and metabolic processes; and, due to its position in these signaling pathways, PI3K is poised to regulate responses that may predispose to malignancies if over-activation or mutation in signaling components arises. Compounds to selectively inhibit components of the PI3K pathway are under development as a cancer therapeutic avenue. For example, a dual inhibitor of p110α and mTOR blocks proliferation of glioma and other tumor cells *in vitro* and in xenograft models *in vivo* [88, 239]. Development of multiple inhibitors for the signaling constituents in the PI3K cascade that are deregulated in cancer and an understanding of how *H. pylori* alters these components is critical for designing customized cancer therapies.

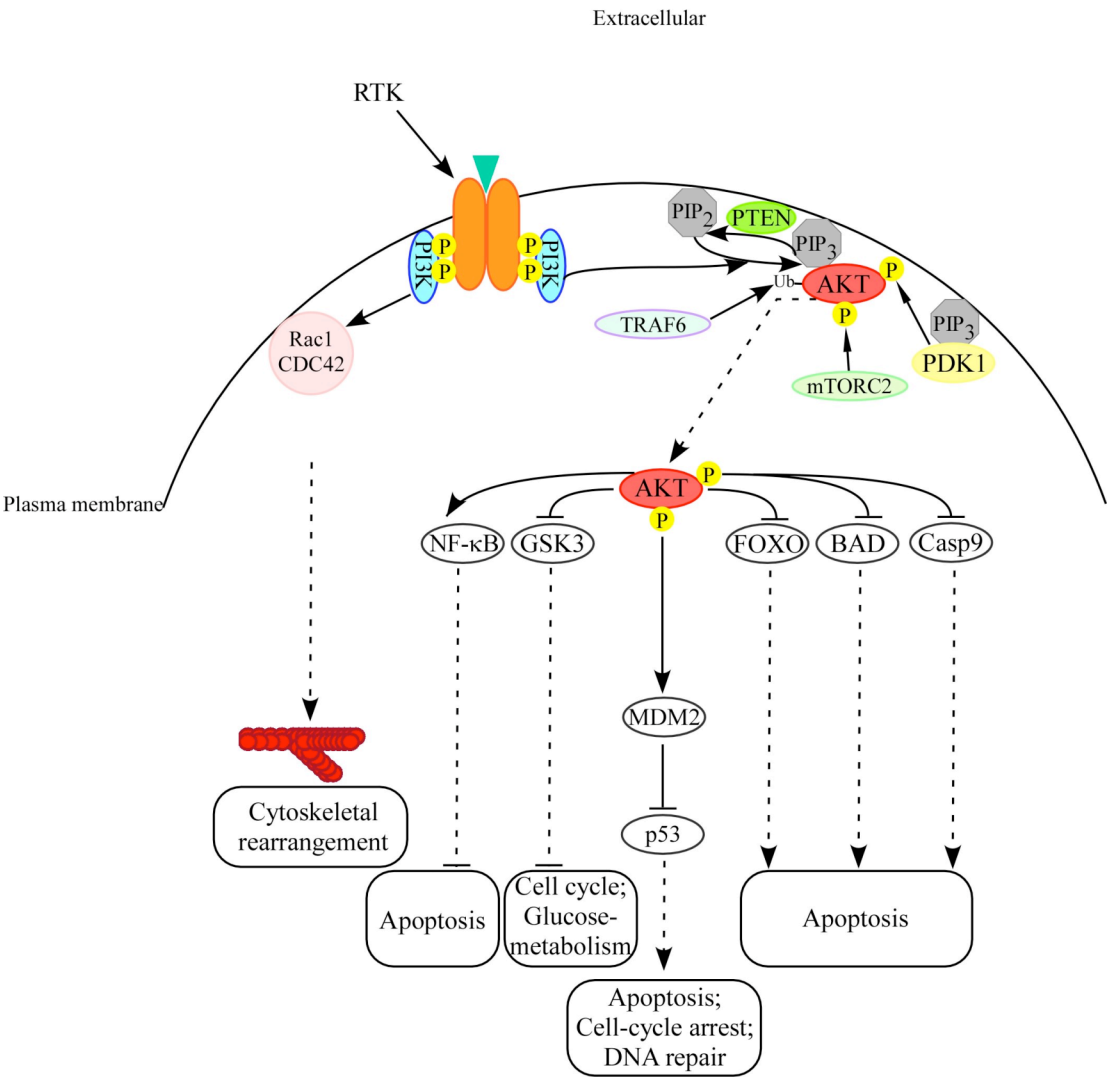

Figure 6. PI3K regulates signaling cascades that participate in carcinogenesis.

Activation of β-catenin and gastric cancer

One specific molecule that may influence *H. pylori*-induced epithelial responses with carcinogenic potential is β-catenin. Membrane-bound β-catenin is a component of adherens junctions that link cadherin receptors to the actin cytoskeleton, and in non-transformed epithelial cells, β-catenin is primarily localized to E-cadherin complexes [308]. Cytoplasmic β-catenin is a downstream component of the Wnt signal transduction pathway. In the absence of Wnt ligands, β-catenin is bound in the cytosol by a multi-protein inhibitory complex, which includes GSK-3β, the adenomatous polyposis coli (APC) tumor suppressor protein, and axin [308]. GSK-3β constitutively phosphorylates β-catenin, targeting it for ubiquitination by E3-SCFβ^{TrCP} and subsequent degradation by the proteosomal complex [308]. Binding of Wnt ligand to it's receptor Frizzled, activates Dishevelled (Dsh) and Wnt co-receptors LRP-5 and LRP-6, which interact with members of the inhibitory complex, leading to inhibition of axin and the kinase activity of GSK-3β (**Figure 7**) [308]. AKT-dependent phosphorylation of GSK-3β at Serine 9 also inhibits its kinase activity [164]. Together these events block β-catenin degradation, leading to its nuclear accumulation, the formation of heterodimers with LEF/TCF transcription complex, and transcriptional activation of genes that regulate normal cellular processes as well as influence carcinogenesis.

Numerous studies have implicated aberrant β-catenin signaling in carcinogenesis. In colorectal carcinoma specimens, inactivating mutations of APC or axin are present in 70-75% of cases [150]. Increased β-catenin expression, as well as mutations within APC,

are also present in gastric adenocarcinoma specimens compared to non-transformed gastric mucosa [311]. Nuclear accumulation of β-catenin is increased within gastric adenomas and foci of dysplasia, suggesting aberrant activation of β-catenin precedes the development of gastric adenocarcinoma [50, 56, 84, 149]. *H. pylori* increases the expression of β-catenin target genes that influence proliferation, apoptosis, and carcinogenesis, such as *c-myc, cyclin D, mmp-7, cox-2,* and *gastrin* within colonized mucosa and during co-culture with gastric epithelial cells *in vitro* [17, 29-31, 69, 74, 106, 129, 179, 187, 201, 255, 263, 273, 303, 339, 345].

Within the context of *H.* pylori infection, translocated CagA has been demonstrated to physically interact with E-cadherin leading to destabilization of the E-cadherin/β-catenin complex and release of β-catenin to the cytoplasm and nucleus (**Figure 7**) [159]. In addition, recent reports now demonstrate that *H. pylori* can also activate β-catenin via mechanisms that involve PI3K-dependent inactivation of GSK-3β, though the specific bacterial factors required for these events remain unclear (**Figure 7**) [200, 297]. Since β-catenin is aberrantly activated by *H. pylori* contact with gastric epithelial cells, is over-expressed within *H. pylori*-associated pre-malignant and malignant lesions, and regulates the transcription of genes that have been implicated in tumor initiation and promotion, it is likely that activation of β-catenin signaling is a central component in regulation of epithelial responses to *H. pylori* that may lower the threshold for carcinogenesis.

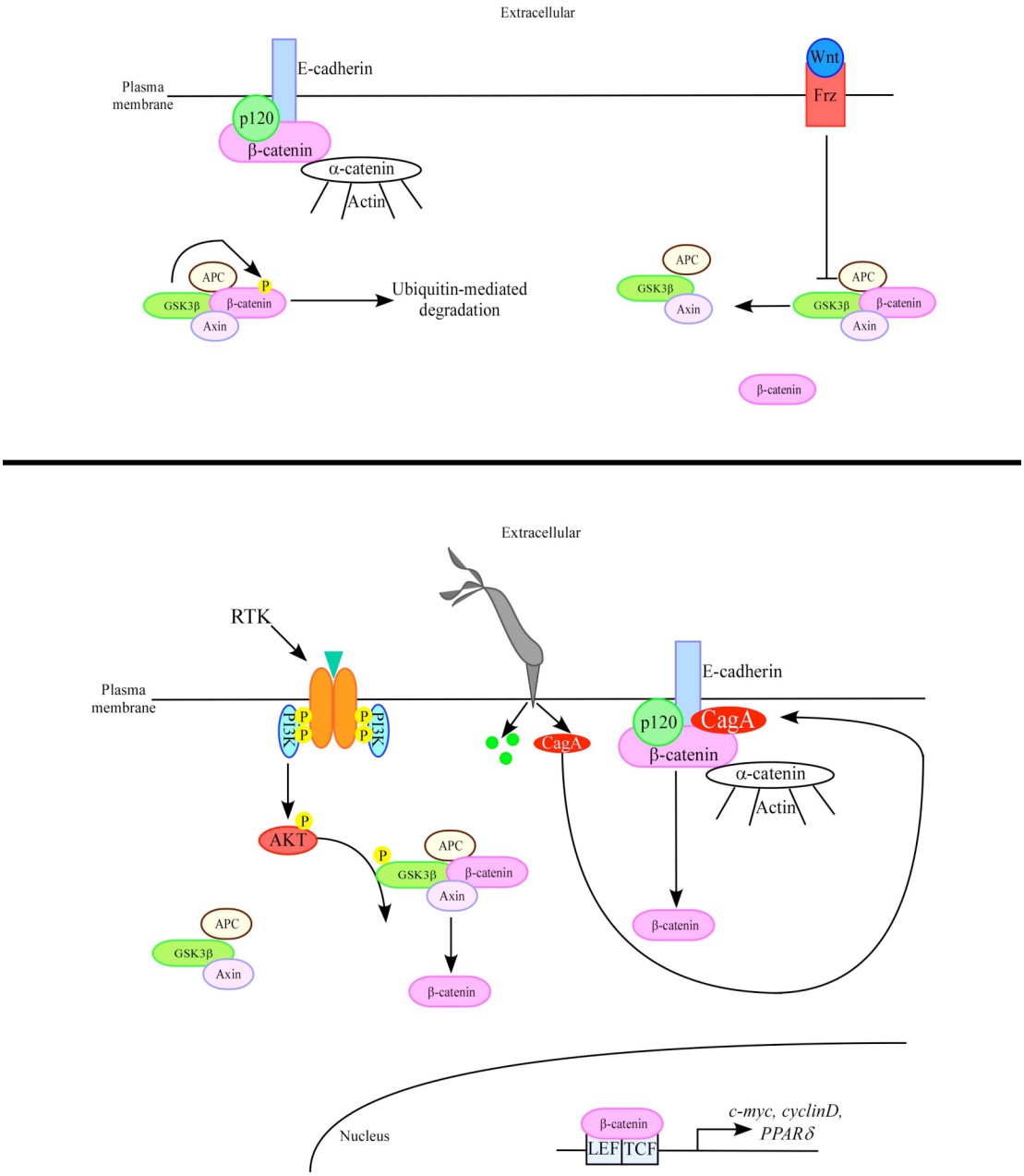

Figure 7. Hypothetical model of β-catenin signaling in unstimulated, WNT-activated, (Panel A) or *H. pylori*-infected (Panel B) gastric epithelial cells.
●, peptidoglycan

p120 and carcinogenesis

p120 is a member of the catenin family, which modulates the function of cadherins [243, 244, 246, 247, 274, 290]. E-cadherin acts as a cell-cell adhesion molecule in epithelial tissues and its turnover is regulated by binding of p120 to the cadherin juxtamembrane domain (**Figure 8**) [11, 12, 73, 137, 244, 246, 247, 274, 290, 305]. p120 is phosphorylated at multiple sites by Src, receptor tyrosine kinases, and ligand-receptor pathways, which include protein kinase C (PKC)- and EGFR-dependent pathways, both of which are activated by *H. pylori* [146, 173, 206, 342].

Reduced membrane expression or aberrant localization of p120 to the cytosol or nucleus has been observed in several epithelial malignancies, including gastric cancer [140, 142, 178, 306]. Loss of E-cadherin or overexpression of p120 *in vitro* results in mislocalization of p120 to the cytoplasm where it can promote motility and metastasis via interactions between p120 and Rho GTPases [11, 118, 204].

p120 is normally present at low levels in the nuclei of non-transformed cells, but is increased within the nuclei of tumor cells [178, 262, 334]. Recently, *H. pylori* infection has been associated with mislocalization of p120 to the nucleus in human gastric epithelium and in infected human primary gastric epithelial cells [156]. Nuclear p120 relieves transcriptional repression exerted by Kaiso, a member of the broad complex, tramtrak, bric a brac/pox virus and zinc finger (BTB/POZ) family [70]. Kaiso acts as a dual specificity repressor that recognizes both sequence-specific consensus sites

(CTGCNA) and methylated CpG nucleotides [71, 217, 236]. The Kaiso/p120 complex coordinately regulates expression of several genes implicated in carcinogenesis such as *c-myc, cyclin D1,* and *mmp-7* (**Figure 8**) [217, 289], all of which are up-regulated by *H. pylori in vitro* and *in vivo* [17, 31, 69, 74, 129, 187, 201, 272, 339, 345]. p120 and Kaiso also mediate expression of additional β-catenin target genes including peroxisome proliferator-activated receptor δ (PPARδ) [217].

Peroxisome proliferator-activated receptor δ (PPARδ)

Peroxisome proliferator-activated receptor δ (PPARδ) and the related isoforms PPARα and PPARγ, constitute a family of ligand-activated transcription factors that are members of the nuclear hormone receptor superfamily [324]. These nuclear receptors are activated by natural ligands, including fatty acids and cholesterol metabolites. PPARs form functional heterodimers with the retinoid X receptor (RXR) and regulate the transcription of target genes that mediate fatty acid oxidation and glucose utilization (**Figure 9**) [32]. PPARδ is expressed in a wide range of tissues, including the gastrointestinal tract, and mouse models have demonstrated a critical role for PPARδ in embryonic development. PPARδ null mice show a high degree of embryonic death, partially due to defects in the placenta and wound healing processes [27, 185, 198, 230]. Indeed, PPARδ has also been demonstrated to be critical for the survival of keratinocytes by upregulating PI3K-AKT signaling [78]. In addition to its roles in normal developmental processes, recent evidence suggests that cross regulation between Wnt/β-catenin/TCF signaling and PPARδ modulates both normal and pathological processes in humans [196].

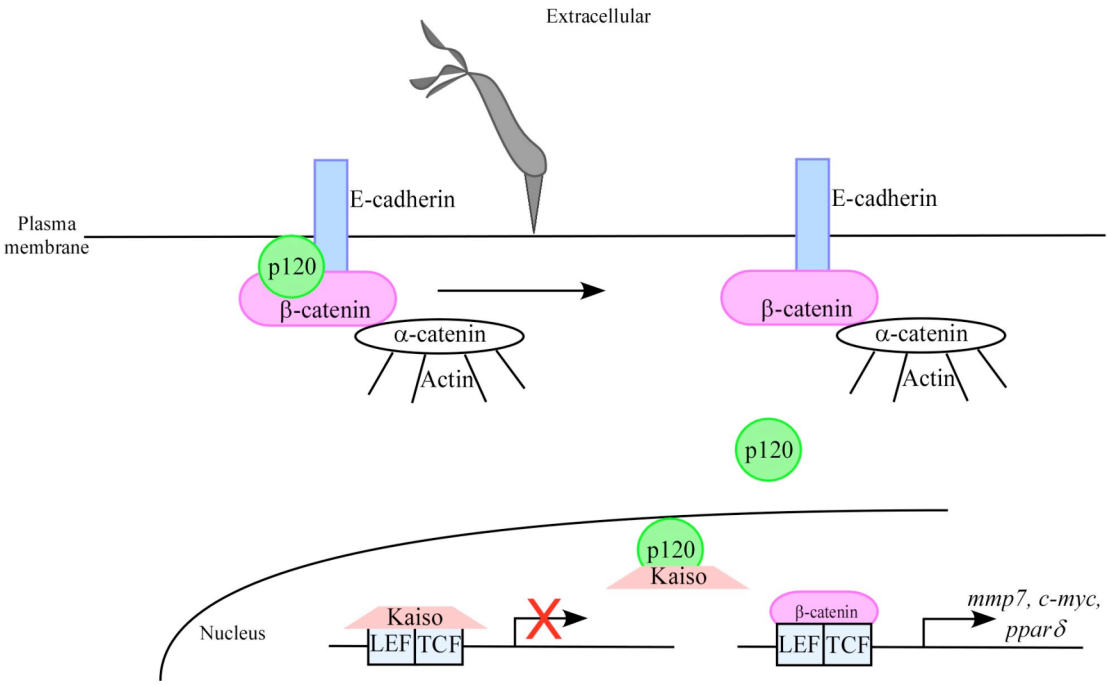

Figure 8. p120 stabilization of adherens junctions. Nuclear accumulation of p120 in response to *H. pylori* infection relieves Kaiso-mediated transcriptional repression of β-catenin target genes.

Genetic and pharmacological studies have revealed important roles for PPARδ in regulating lipid metabolism and energy homeostasis. Overexpression of PPARδ in mouse adipose tissue inhibits hyperlipidemia, steatosis, and obesity that is either genetically-induced or induced by high-fat diet [328]. Additionally, PPARδ null mice exhibit an obese phenotype [328]. A number of high affinity synthetic ligands for PPARδ are currently available and pharmacologic studies have demonstrated that the PPARδ selective agonist GW501516 increases HDL-cholesterol while lowering triglyceride levels and insulin in obese rhesus monkeys [212]. GW501516 is now being used in Phase III clinical trials to evaluate its efficacy for treatment of patients with hyperlipidemias and obesity. However, recent studies in animal models have demonstrated that PPARδ agonists may promote carcinogenesis [230, 240].

Levels of PPARδ are elevated in most human colorectal cancers as well as carcinomas that develop in murine models of colon cancer including $Apc^{Min/+}$ mice and azoxymethane (AOM)-treated mice, and the PPARδ agonist GW501516 has been shown to be proneoplastic in mice [120]. However, two studies reported that disruption of PPARδ increased polyp formation in $Apc^{Min/+}$ mice in the absence of exogenous PPARδ stimulation, which has necessitated more in-depth studies [123, 240]. Conversely, it has recently been demonstrated that deletion of PPARδ decreases intestinal adenoma growth in $Apc^{Min/+}$ mice and inhibits the tumor promoting effects of GW501516 [324]. In another study using a xenograft model, disruption of both PPARδ alleles in human HCT-116 colon carcinoma cells decreased tumorigenicity, indicating that activation of PPARδ promotes tumor growth [216]. The PPARδ agonist GW501516 has been shown to

stimulate proliferation of human breast, prostate, and hepatocellular carcinoma cells [112, 293] and in a murine mammary tumor model, treatment with GW501516 accelerated tumor formation [349]. These results are concordant with recent observations that a target gene which is transcriptionally activated by PPARδ, cyclin E1, is a cell cycle regulatory protein that, in association with Cdk2, drives cells from G1 into S phase via hyperphosphorylation of the retinoblastoma protein Rb [351].

Prostaglandin PGI_2, a metabolite of cyclooxygenase-2, is one endogenous PPARδ ligand [92, 151]. Cyclooxygenase (COX) enzymes are encoded by two distinct genes that catalyze key steps in the formation of prostaglandins. COX-1 is constitutively expressed in the gastrointestinal tract, while COX-2 can be induced by a variety of stimuli including *H. pylori* [335]. Levels of COX-2 are increased within gastric mucosa of *H. pylori*-infected individuals, suggesting that prostaglandins that activate PPARδ may be oncogenic [106, 179, 250, 263, 296, 303, 314]. Since our previous data have demonstrated that *H. pylori* can activate host effectors (β-catenin, p120/Kaiso) that also regulate expression of PPARδ, we hypothesized that up-regulation of PPARδ mediates pathogenic outcomes that develop in response to *H. pylori.*

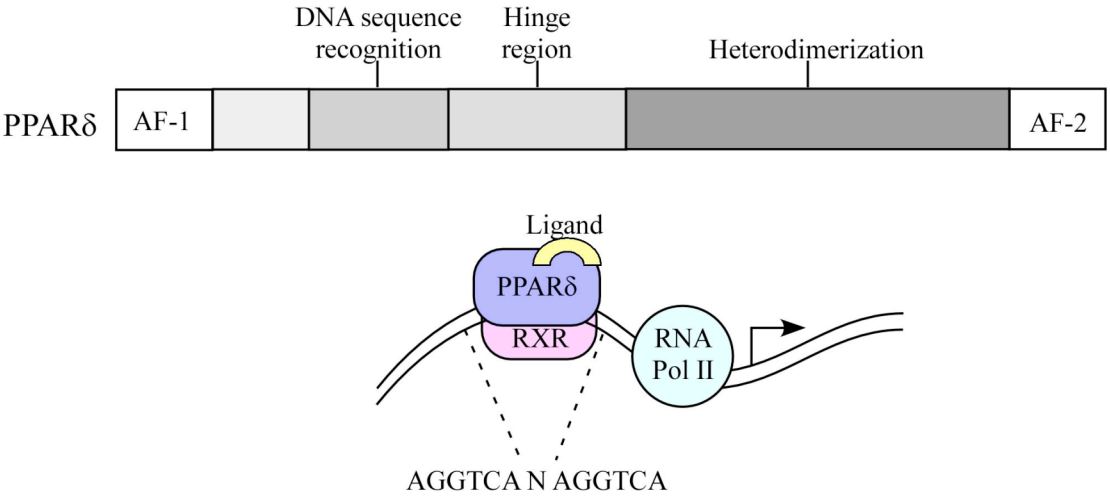

Figure 9. PPARδ (Upper panel) in conjunction with RXR (Lower panel) regulates transcription of genes that influence cell responses in carcinogenesis. AGGTCA N AGGTCA, the PPARδ response element in the promoter of target genes.

Summary and dissertation goals

Gastric adenocarcinoma is strongly associated with the presence of *H. pylori*. Microbial factors of *H. pylori* and host responses induced by the interactions of *H. pylori* with gastric epithelial cells play important roles in the development of disease. PI3K/AKT and β-catenin/p120 are multifunctional host proteins that coordinate carcinogenic epithelial responses when aberrantly activated, such as in malignant gastric lesions. In Chapter II, we demonstrate that *H. pylori* infection results in upregulation of PI3K-AKT signaling, through stimulation of EGFR. Activation of this pathway reduces rates of epithelial cell death induced by *H. pylori* and promotes resistance to apoptosis. In Chapter III, we demonstrate that *H. pylori* infection induces additional host signaling pathways to potentiate a proliferative response in gastric epithelial cells. Specifically, PPARδ, a target of β-catenin transcriptional activation, contributes to increased rates of gastric epithelial cell proliferation in response to *H. pylori* infection. Based on these findings we hypothesize that an anti-apoptotic response in the presence of increased proliferation increases the risk of retaining mutagenized gastric epithelial cells in the presence of *H. pylori* induced gastritis. Taken together, these studies have identified effectors that directly mediate host responses related to carcinogenesis. Molecular delineation of such pathways activated by host-microbial interactions will improve our understanding of *H. pylori*-induced carcinogenesis, allowing for targeted therapies to high-risk individuals, as well as provide insight into other malignancies that arise within the context of pathogen-induced inflammation.

CHAPTER II

ACTIVATION OF PI3K BY *HELICOBACTER PYLORI* PROMOTES RESISTANCE TO APOPTOSIS

Summary

Helicobacter pylori is the strongest identified risk factor for gastric adenocarcinoma. One *H. pylori* virulence constituent that augments cancer risk is the *cag* secretion system, which translocates CagA and peptidoglycan into host cells, eventuating in activation of signal transduction pathways. AKT is a target of phosphatidylinositol 3-phosphate kinase (PI3K) and is activated in gastric cancer, but the relationship between PI3K-AKT and *H. pylori*-induced cellular responses with carcinogenic potential remains unclear. We defined the molecular pathways mediating *H. pylori*-stimulated AKT activation and the biological consequences of these events in gastric epithelial cells. *H. pylori* enhanced PI3K-AKT signaling in a Src and EGFR-dependent manner, which was also mediated by a functional *cag* secretion system, and peptidoglycan. PI3K activation attenuated apoptosis in response to *H. pylori* infection. These results indicate that PI3K-AKT signaling regulates pathophysiologic responses to *H. pylori* that may lower the threshold for carcinogenesis.

Introduction

Chronic gastritis induced by *Helicobacter pylori* persists for decades and increases the risk of gastric adenocarcinoma [21, 209]. Although *H. pylori*-induced gastritis is the strongest known risk factor for gastric cancer, only a fraction of colonized individuals ever develop neoplasia, and enhanced cancer risk is mediated by strain-specific bacterial factors and/or inflammatory responses governed by host genetic diversity. The *cag* pathogenicity island (*cag* PAI) is a virulence locus present in approximately 60% of U.S. *H. pylori* strains [128, 268], and strains that harbor the *cag* PAI (cag^+) significantly augment the risk for distal gastric cancer compared to strains that lack the *cag* island (cag^-) [10, 97].

Several *cag* genes, such as *cagE,* encode components of a type IV secretion system that exports bacterial proteins into host cells. The terminal product of the *cag* island, CagA, is translocated into gastric epithelial cells following bacterial attachment [320]. CagA subsequently undergoes tyrosine phosphorylation by Src and Abl kinases, and phospho-CagA alters gastric cell morphology and aberrantly activates signaling molecules such as SHP-2 [252]. Unphosphorylated CagA can also exert effects within host cells such as alteration of cell polarity and activation of β-catenin, responses that have been implicated in carcinogenesis [213]. In addition to CagA, components of peptidoglycan can be translocated into host cells by the *cag* secretion system where they are sensed by the intracellular pattern recognition receptor Nod1, which activates NF-κB and induces production of pro-inflammatory cytokines such as IL-8 [101].

Signal transduction pathways activated in response to bacterial contact play an important role in *H. pylori* pathogenesis. Phosphatidylinositol 3-kinase (PI3K) is an integral component of a signal transduction pathway that regulates host cellular responses altered in tumorigenesis. PI3K signaling can be activated by ligand-dependent activation of receptor tyrosine kinases such as EGFR [153]. Src kinases, acting both downstream and upstream of EGFR, can also activate PI3K signaling [177]. PI3K activation results in stimulation of phosphatidylinositol-dependent kinase 1 (PDK1), a kinase that phosphorylates and activates AKT [72]. AKT mediates the downstream effects of PI3K by phosphorylating multiple targets that regulate diverse cellular functions including proliferation and survival. PI3K-AKT signaling is increased in gastric cancer specimens and enhanced levels of AKT phosphorylation correlate with advanced stages of disease [43]. Thus, PI3K is well positioned to regulate epithelial responses that may predispose to malignancies.

Cell survival is one host response that is regulated by PI3K and AKT activation [169, 228]. AKT-dependent phosphorylation of pro-apoptotic Bcl-2 homology domain 3 (BH3)-only proteins (Bad, Bik, Bid, Bim, Noxa) inhibits their activity [222], while AKT-dependent phosphorylation of BCL-2 proteins activates their pro-surivial signals [172]. Pro-caspase 9 can undergo inhibitory phosphorylation by AKT [189], which attenuates apoptosis, thereby promoting cell survival and enhancing the susceptibility of cells to mutagenesis. Since *H. pylori* increases cell proliferation and attenuates apoptosis in humans and in rodent models of infection [147, 213, 252, 291], we determined the ability of *H. pylori* to activate PI3K-AKT signaling in gastric epithelial cells and investigated the

molecular pathways mediating these events to define potential tumor-promoting responses toward this pathogen.

Experimental Procedures

Cell Culture and Reagents.

AGS or MKN28 human gastric epithelial cells were grown in RPMI medium 1640 (GIBCO/BRL) with 10% FBS (Sigma) and 20 μg/ml gentamicin (GIBCO/BRL) under 5% CO_2 air at 37°C. Pharmacological inhibitors LY294002 (Cell Signaling Technology), AG1478 (Calbiochem), PP2 (Calbiochem), SU6656 (Calbiochem), AG1295 (Calbiochem), and STI-571 (LC Laboratories) were used at concentrations of 50 μM, 600 nM, 10 μM, 2μM, 50μM and 10μM, respectively. For Western immunoblot and flow cytometry analysis, AGS cells were plated at 5 x 10^5 cells/well in 6-well plates in 2 mL culture medium.

H. pylori strains.

The *H. pylori* cag^+ rodent-adapted strain 7.13, the cag^+ clinical strain J166, or the cag^- clinical isolate J68, were grown in *Brucella* broth with 5% FBS for 18 hours, harvested by centrifugation, and were added to gastric cells at a bacteria-to-cell ratio of 100:1. Isogenic $cagA^-$, $cagE^-$, and slt^- null mutants were constructed within strain 7.13 by insertional mutagenesis using *aphA* and were selected with kanamycin (25 μg/ml) as described previously [184]. *H. pylori* were heat-killed by boiling at 100°C for 10 minutes, while *H. pylori* filtrates were prepared by passing broth supernatants through a 0.2 μM pore-size filter (Corning).

Western Blot Analysis.

Gastric cell lysates were harvested in lysis buffer (50 mM Tris pH 7.2, 150 mM NaCl, 1% Triton X-100, 0.1% SDS) including protease and phosphatase inhibitors (Sigma). Proteins (30 μg) were separated by 10% SDS-PAGE and transferred to poly-vinylidene difluoride membranes (Pall). Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBST), incubated for 24 hours with a purified rabbit polyclonal anti-phospho-AKT (Ser473) antibody (1:1000 dilution; Cell Signaling), a rabbit polyclonal anti-total AKT antibody (1:1000 dilution; Cell Signaling), a monoclonal mouse anti-GAPDH antibody (1:2000 dilution; Santa Cruz Biotech), a mouse monoclonal anti-phospho-tyrosine-99 antibody (1:300 dilution; Santa Cruz Biotech), a rabbit polyclonal anti-CagA antibody (1:5000 dilution; Austral Biologicals), a rabbit polyclonal anti-phospho-Src family antibody (1:1000 dilution; Cell Signaling), a rabbit polyclonal anti-Src antibody (1:1000 dilution; Cell Signaling), a mouse monoclonal anti-phospho-EGFR (Tyr1068) antibody (1:1000; Millipore), a rabbit polyclonal anti-EGFR antibody (1:3000; Millipore), a rabbit polyclonal anti-phospho-Gab1 antibody (1:1000; Cell Signaling), or a rabbit polyclonal anti-Gab1 antibody (1:1000 dilution; Cell Signaling). Goat anti-rabbit (1:5000 dilution; Santa Cruz Biotech) or goat anti-mouse (1:5000 dilution; Santa Cruz Biotech) horseradish peroxidase-conjugated secondary antibodies were used followed by enhanced chemiluminescence detection following the manufacturer's instructions (Perkin Elmer). Immunoblots were quantified with the GeneTools Software (Syngene).

Flow Cytometry Analysis.

AGS cells co-cultured with *H. pylori* were washed with PBS and harvested using 0.25% Trypsin/EDTA (GIBCO/BRL). Cells were collected by centrifugation and resuspended in binding buffer (10x: 0.1 M HEPES pH 7.4, 1.4 M NaCl, 25 mM $CaCl_2$) at a concentration of 5 x 10^5 cells/mL. Cells were stained with Annexin V-APC (BD Bioscience) and Propidium iodide/RNAse (BD Bioscience) and were analyzed by quantitative flow cytometry.

Apoptotic Resistance Assays.

AGS cells were infected with *H. pylori* for 3 hours followed by incubation with 50 μM LY294002 for 1 hour. After a four hour exposure to 1 μM Staurosporine (Sigma-Aldrich), cells were harvested for Annexin V-flow cytometry analysis as described [147].

Transient transfection of siRNA.

AGS cells (2.5 x 10^5) in 6-well plates were transiently transfected using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Briefly, transfection reagent (5.0 μl/well) was mixed with siRNA oligos (10 μl of 10 μM solution/well) in 500 μl Opti-MEM (Life Technologies). Cells were incubated with the transfection mixture for 24 hours, fresh medium was added, and bacterial co-cultures were performed 24 hours later.

Statistical Analysis.

All experiments were performed on at least three independent occasions. Statistical analysis was performed by Student's t test and ANOVA using Prism Graph Pad. A P-value < 0.05 was defined as statistically significant.

Results

H. pylori activates PI3K-AKT signaling in gastric epithelial cells.

We used a *cag*$^{+}$ *H. pylori* strain, 7.13, that reproducibly causes gastric cancer in rodent models, to determine whether *H. pylori* alters AKT activity. AGS cells were infected with strain 7.13 at a multiplicity of infection (MOI) of 100 or were exposed to medium alone. *H. pylori* strain 7.13 increased AKT phosphorylation at serine 473 compared to uninfected controls at each time point (**Figure 10**). Similar patterns of AKT activation were observed following infection of AGS cells with the *cag*$^{+}$ human clinical isolate J166 or infection of MKN28 human gastric epithelial cells with strains 7.13 or J166 (data not shown).

Inactivation of cagE abolishes AKT activation by H. pylori.

We next investigated the role of bacterial factors in PI3K-AKT signaling events. Neither heat-killed bacteria nor soluble factors contained in *H. pylori* filtrates stimulated phosphorylation of AKT, indicating that viable *H. pylori* are required for AKT activation (**Figure 11A, 11B**). The *cag* secretion system encodes several proteins that affect cellular signaling after live *H. pylori* have bound host cells. To define the role of *cag* components in AKT activation, AGS cells were incubated with the *H. pylori cag*$^{+}$ strain 7.13 or its isogenic *cagA*$^{-}$ or *cagE*$^{-}$ null mutant derivatives. AKT activation was significantly decreased in cells incubated with the 7.13 *cagE*$^{-}$, but not the *cagA-,* mutant versus the wild-type strain (**Figure 11C, 11D**). Similarly, the *cag*$^{-}$ clinical isolate J68

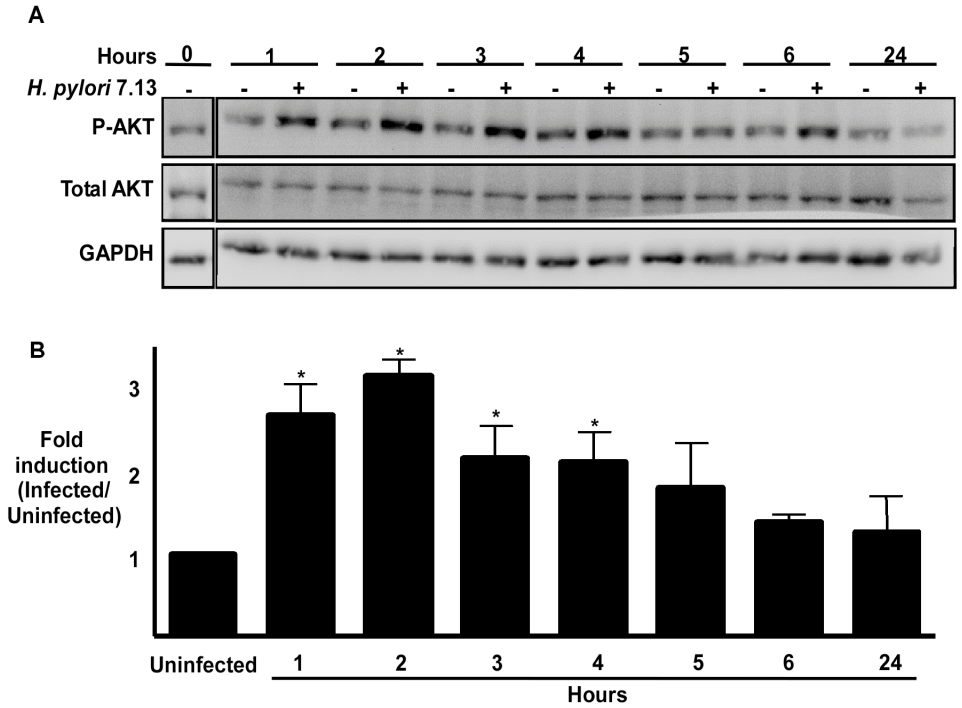

Figure 10. ***H. pylori*** **induces AKT activation** ***in vitro*** **in a time-dependent manner.** ***(A)*** AGS cells were co-cultured with the *H. pylori cag*$^{+}$ strain 7.13 at a bacteria/cell ratio of 100:1. One through twenty-four hours after incubation, whole cell lysates were harvested and subjected to Western blot analysis using an anti-phospho-AKT (Ser473) antibody. (-), cells incubated with medium alone. A representative blot is shown. Western blots for total AKT served as normalization controls for AGS cell viability under different experimental conditions and Western blots for GAPDH served as loading controls. ***(B)*** Densitometric analysis of multiple Western blot repetitions performed on at least 3 occasions. Levels of phospho-AKT were normalized to total AKT and levels were expressed as fold-induction of infected cells compared with uninfected cells at each time point. Error bars = SEM. **P* < 0.01 vs. uninfected control.

failed to induce AKT phosphorylation (data not shown). These findings indicate that a functional *cag* secretion system, but not *cagA,* is required for induction of PI3K-AKT signaling.

Peptidoglycan is required for activation of AKT by H. pylori.

In addition to CagA, peptidoglycan can be translocated by the *cag* secretion system, and can alter host signaling. Therefore, we examined the role of peptidoglycan in AKT activation using a 7.13 isogenic *slt* mutant. The *slt* mutant lacks the soluble lytic transglycosylase (slt) required for peptidoglycan turnover and release. We first established that inactivation of *slt* in strain 7.13 does not alter CagA translocation into host cells (**Figure 12A**). We then co-cultured AGS cells with wild-type strain 7.13 or the isogenic *slt*$^-$ mutant. Cells co-cultured with the *slt*$^-$ mutant contained significantly lower levels of phospho-AKT compared to cells infected by wild-type 7.13 (**Figure 12B, 12C**). These results indicate that peptidoglycan, in conjunction with a functional *cag* secretion system, is required for maximal AKT stimulation by *H. pylori*.

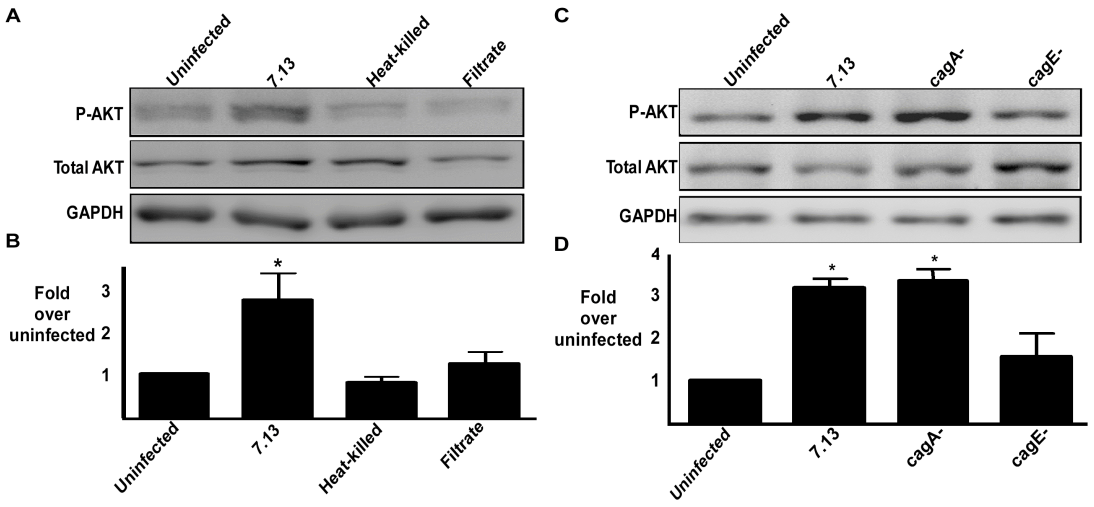

Figure 11. AKT phosphorylation by *H. pylori* is dependent on specific genes within the *cag* pathogenicity island. ***(A)*** AGS cells were incubated in the absence or presence of live *H. pylori* strain 7.13 at a bacteria/cell ratio of 100:1, heat-killed *H. pylori*, or *H. pylori* 7.13 filtrate for two hours. Whole cell lysates were subjected to Western blot analysis using an anti-phospho AKT (Ser473) antibody. Anti-total AKT blots served as normalization controls for AGS cell viability under different experimental conditions and anti-GAPDH blots served as loading controls. ***(B)*** Densitometric analysis of Western blots performed on 3 occasions. Error bars = SEM. **P* <0.04 vs. AGS cells alone. ***(C)*** AGS cells were cultured in the absence or presence of the *H. pylori cag*$^{+}$ strain 7.13 or its isogenic *cagA*$^{-}$ or *cagE*$^{-}$ null mutant derivatives at bacteria/cell ratios of 100:1. Two hours post infection, whole cell lysates were subjected to Western blot analysis using an anti-phospho-AKT (Ser473) antibody. A representative blot is shown. Western blots for total AKT served as normalization controls and Western blots for GAPDH served as loading controls. ***(D)*** Densitometric analysis of multiple Western blot repetitions performed on at least 5 occasions. Error bars = SEM. **P* < 0.002 vs. AGS cells alone.

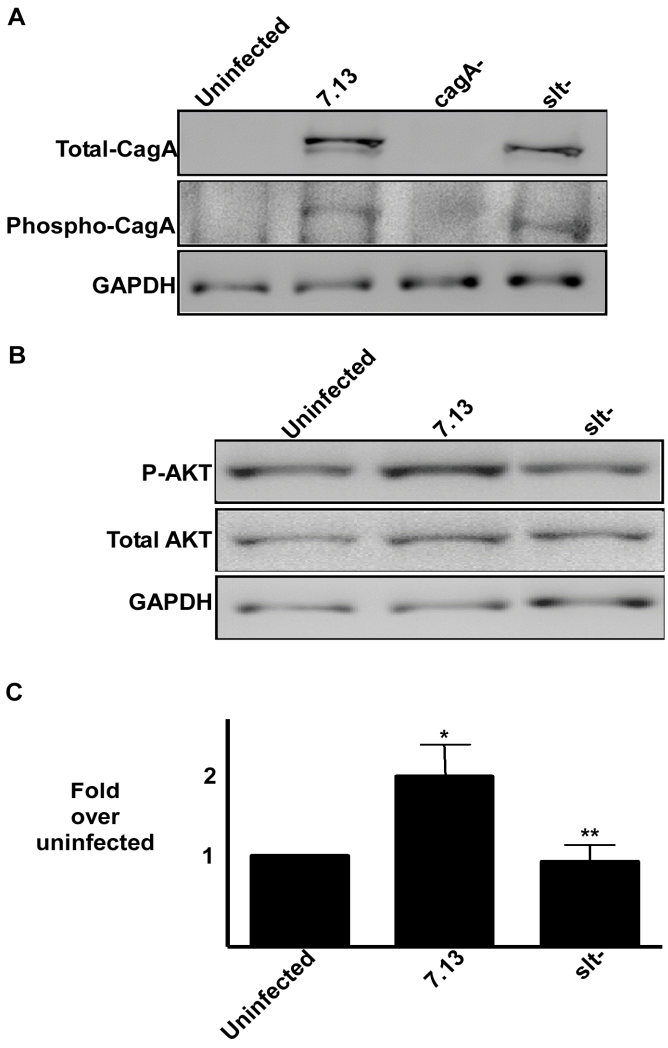

Figure 12. AKT phosphorylation by *H. pylori* is mediated by peptidoglycan. ***(A)*** AGS cells were cultured in the absence or presence of wild-type *H. pylori* strain 7.13 or its isogenic *cagA*$^-$ or *slt*$^-$ null mutant at a bacteria/cell ratio of 100:1. Two hours post infection, whole-cell lysates were subjected to Western blot analysis using an anti-phospho-tyrosine 99 antibody or an anti-CagA antibody. A representative blot is shown. Western blots for GAPDH served as loading controls. ***(B)*** *H. pylori* strain 7.13 or its isogenic *slt* null mutant derivative, were added to AGS cells at a bacteria/cell ratio of 100:1. Two hours after incubation, whole-cell lysates were subjected to Western blot analysis using an anti-phospho-AKT (Ser473) antibody. A representative blot is shown. Western blots for total AKT served as normalization controls for AGS cell viability under different experimental conditions and Western blots for GAPDH served as loading controls. ***(C)*** Densitometric analysis of multiple Western blot repetitions performed on at least 3 occasions. Error bars = SEM. **P* < 0.04 vs AGS cells alone; ***P* < 0.009 vs AGS cells incubated with wild-type *H. pylori*.

H. pylori-induced AKT activation is dependent on activation of PI3K, Src, and EGFR.

AKT activation is regulated by many of the same constituents that are activated by *H. pylori* (e.g., EGFR, Src) [33]. As a prelude to defining the molecular pathways mediating *H. pylori*-induced AKT activation, we first confirmed that our prototype strain could activate EGFR and Src, and also established the efficacy of PI3K, EGFR and Src inhibitors. *H. pylori* strain 7.13 induced phosphorylation of Src and EGFR in AGS cells (**Figure 13A**). AGS cell lysates were then assessed for phospho-AKT after stimulation with EGF, a potent inducer of AKT activation. Each inhibitor was sufficient to attenuate EGF-stimulated AKT activation (**Figure 13B**). AGS cells were then co-cultured with strain 7.13 in the absence or presence of the PI3K inhibitor LY294002 or the Src inhibitor PP2. *H. pylori* alone activated AKT by two hours (**Figure 13C, 13D**). As predicted, AKT activation in response to *H. pylori* was completely abolished by PI3K inhibition (**Figure 13C, 13D**). PI3K-dependent AKT activation was further confirmed using an independent PI3K inhibitor, Wortmannin (200nM) (data not shown).

H. pylori-induced activation of AKT was also dependent on Src, as treatment with the Src inhibitor PP2 blocked AKT activation (**Figure 13C, 13D**). The inhibitor PP2, however, can also exert activity against platelet-derived growth factor receptor (PDGFR) signaling as well as signaling initiated by c-Abl and c-kit [189]. Therefore, we repeated co-culture experiments in the presence or absence of specific inhibitors of these pathways. *H. pylori*-induced AKT activation was unchanged in the presence of PDGFR or c-Abl/c-kit inhibitors (**Figure 13E, 13F**), indicating that Src plays a role in microbial-induced activation of AKT.

EGFR transactivation can mediate PI3K and Src activation and this receptor can be transactivated by *H. pylori* (**Figure 13A**) [163]; therefore we next determined the role of EGFR in *H. pylori*-induced AKT activation. Co-culture of AGS cells with *H. pylori* in the presence of the EGFR kinase inhibitor AG1478 significantly reduced AKT phosphorylation to levels seen in uninfected controls (**Figure 13C, 13D**). However, the EGFR inhibitor AG1478 also inhibits FAK, a component of another PI3K-dependent pathway. Therefore, to rule out involvement of FAK, we determined whether *H. pylori* infection could stimulate Gab1 phosphorylation, an event dependent on EGFR transactivation. As shown in **Figure 13G**, co-culture with wild-type strain 7.13 induced Gab1 phosphorylation. The pattern of Gab1 phosphorylation mirrored AKT activation as *H. pylori* mutant strains that lacked *cagE* or *slt* failed to induce phosphorylation of Gab1 (**Figure 13G**). Finally, to more firmly implicate EGFR and Src signaling in these events, we co-cultured *H. pylori* strain 7.13 with AGS cells in the presence or absence of a Src family kinase inhibitor that does not activate PDGFR (SU6656) [133]. As demonstrated in **Figures 13E** and **13F**, pre-incubation with SU6656 attenuated the ability of *H. pylori* to activate AKT. Collectively, these results indicate that transactivation of EGFR and Src activation are likely required for *H. pylori*-induced AKT activation.

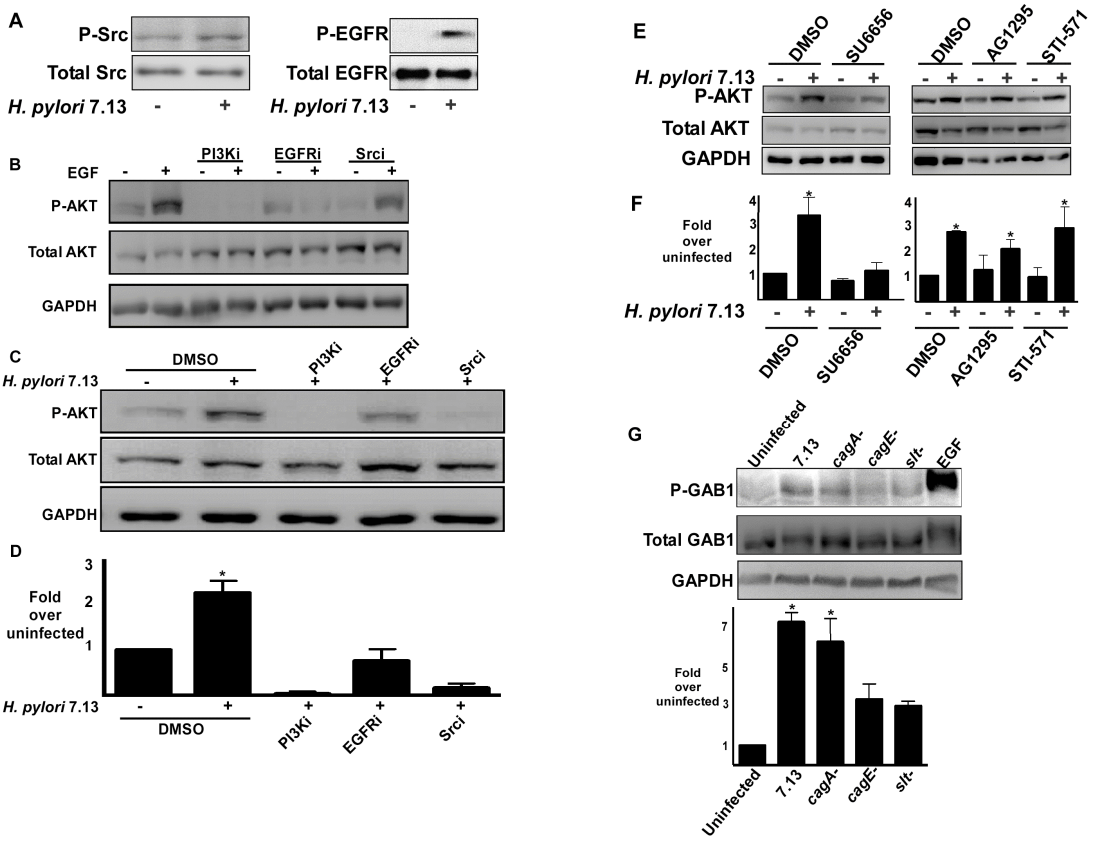

Figure 13. *H. pylori*-induced AKT phosphorylation in AGS cells is dependent on activation of PI3K, EGFR, and Src. ***(A)*** *H. pylori* strain 7.13 was added to AGS cells at a bacteria/cell concentration of 100:1. Two hours post-infection, whole cell lysates were subjected to Western blot analysis using an anti-phospho-Src or an anti-phospho-EGFR antibody. (-), cells incubated with medium alone. A representative blot is shown. Western blots for total Src or EGFR served as normalization controls for AGS cell viability under different experimental conditions. ***(B)*** AGS cells were incubated with the PI3K inhibitor LY294002 (50 μmol/L), EGFR kinase inhibitor AG1478 (600 nmol/L), or Src inhibitor PP2 (10 μmol/L) for one hour prior to EGF exposure for 15 minutes. Levels of phospho- (Ser473) and total AKT were determined by Western blot analysis of whole cell lysates. ***(C)*** *H. pylori* strain 7.13 was added to AGS cells at a bacteria/cell concentration of 100:1 in the absence or presence of vehicle alone (DMSO), or 50 μmol/L LY294002, 600 nmol/L AG1478, or 10 μmol/L PP2. Two hours post-infection, whole cell lysates were subjected to Western blot analysis using an anti-phospho-AKT antibody. (-), cells incubated with medium alone. A representative blot is shown. Western blots for total AKT served as normalization controls for AGS cell viability under different experimental conditions and Western blots for GAPDH served as loading controls. ***(D)*** Densitometric analysis of multiple Western blot repetitions performed on at least 3 occasions. Error bars = SEM. *P < 0.0001 vs. AGS cells alone. ***(E)*** H. pylori strain 7.13 was added to AGS cells at a bacteria/cell concentration of 100:1 in the absence or presence of vehicle alone (DMSO), or 2 μmol/L SU6656, 50 μmol/L AG1295, or 10 μmol/L STI-571. Two hours post-infection, whole cell lysates were

subjected to Western blot analysis using an anti-phospho-AKT (Ser473) antibody. (-), cells incubated with medium alone. A representative blot is shown. Western blots for total AKT served as normalization controls for AGS cell viability under different experimental conditions and Western blots for GAPDH served as loading controls. ***(F)*** Densitometric analysis of multiple Western blot repetitions performed on at least 3 occasions. Error bars = SEM. *P < 0.04 vs. AGS cells alone. ***(G)*** AGS cells were cultured in the absence or presence of the *H. pylori* cag^+ strain 7.13 or its isogenic $cagA^-$, $cagE^-$, or slt^- null mutant derivatives at bacteria/cell ratios of 100:1. Two hours post infection, whole cell lysates were subjected to Western blot analysis using an anti-phospho-Gab1 antibody. EGF was used as a positive control for Gab1 phosphorylation and was added for 15 minutes. A representative blot is shown. Western blots for total Gab1 served as normalization controls and Western blots for GAPDH served as loading controls. Densitometric analysis of multiple Western blot repetitions performed on at least 3 occasions is shown below representative Western blot. Error bars = SEM. *P < 0.02 vs. AGS cells alone.

Activation of AKT by H. pylori attenuates apoptosis and promotes cell survival.

Because AKT activation by PI3K attenuates apoptosis, we next determined the contribution of PI3K signaling to *H. pylori*-mediated apoptosis. AGS cells co-cultured with strain 7.13 in the absence or presence of the PI3K inhibitor LY294002 or vehicle control were stained with Annexin V and Propidium iodide for analysis of apoptosis using flow cytometry. As expected, treatment with the PI3K inhibitor alone induced a small population of uninfected cells into early apoptosis. Co-culture of AGS cells with *H. pylori* increased apoptosis, but this phenotype was significantly enhanced in the presence of the PI3K inhibitor (**Figure 14A, 14B**).

To more robustly demonstrate that PI3K-AKT signaling regulates *H. pylori*-induced cell survival, we transiently transfected AGS cells with scrambled or AKT-specific siRNA. Western blot analysis indicated that AKT expression was significantly reduced using AKT-specific, but not scrambled, siRNA (**Figure 14C**). *H. pylori* strain 7.13 was then co-cultured with AKT-deficient or wild-type control AGS cells and apoptosis was assessed using flow cytometry. Similar to results obtained using a chemical inhibitor of PI3K, inhibition of AKT significantly augmented the ability of *H. pylori* to induce apoptosis (**Figures 14D, 14E**). These data indicate that activation of PI3K-AKT promotes gastric cell survival in the presence of *H. pylori*.

A recent study demonstrated that *H. pylori* can not only induce apoptosis, but can also promote resistance to this phenotype in response to a known apoptosis-inducing agent, Staurosporine (Stsp) [320]. To extend our data implicating PI3K-AKT in cell survival,

we evaluated the ability of PI3K to promote apoptotic resistance in *H. pylori*-infected AGS cells. Cells were infected with *H. pylori* strain 7.13 and then treated with Stsp to induce apoptosis. Inhibition of PI3K did not significantly alter apoptosis in uninfected cells exposed to Stsp (**Figure 14F**). AGS cells infected with *H. pylori* were more resistant to Stsp-induced apoptosis than cells pre-treated with medium alone (**Figure 14F**). However, inhibition of PI3K attenuated the anti-apoptotic activity of *H. pylori*, further supporting a role for *H. pylori*-induced PI3K signaling in promoting cell survival.

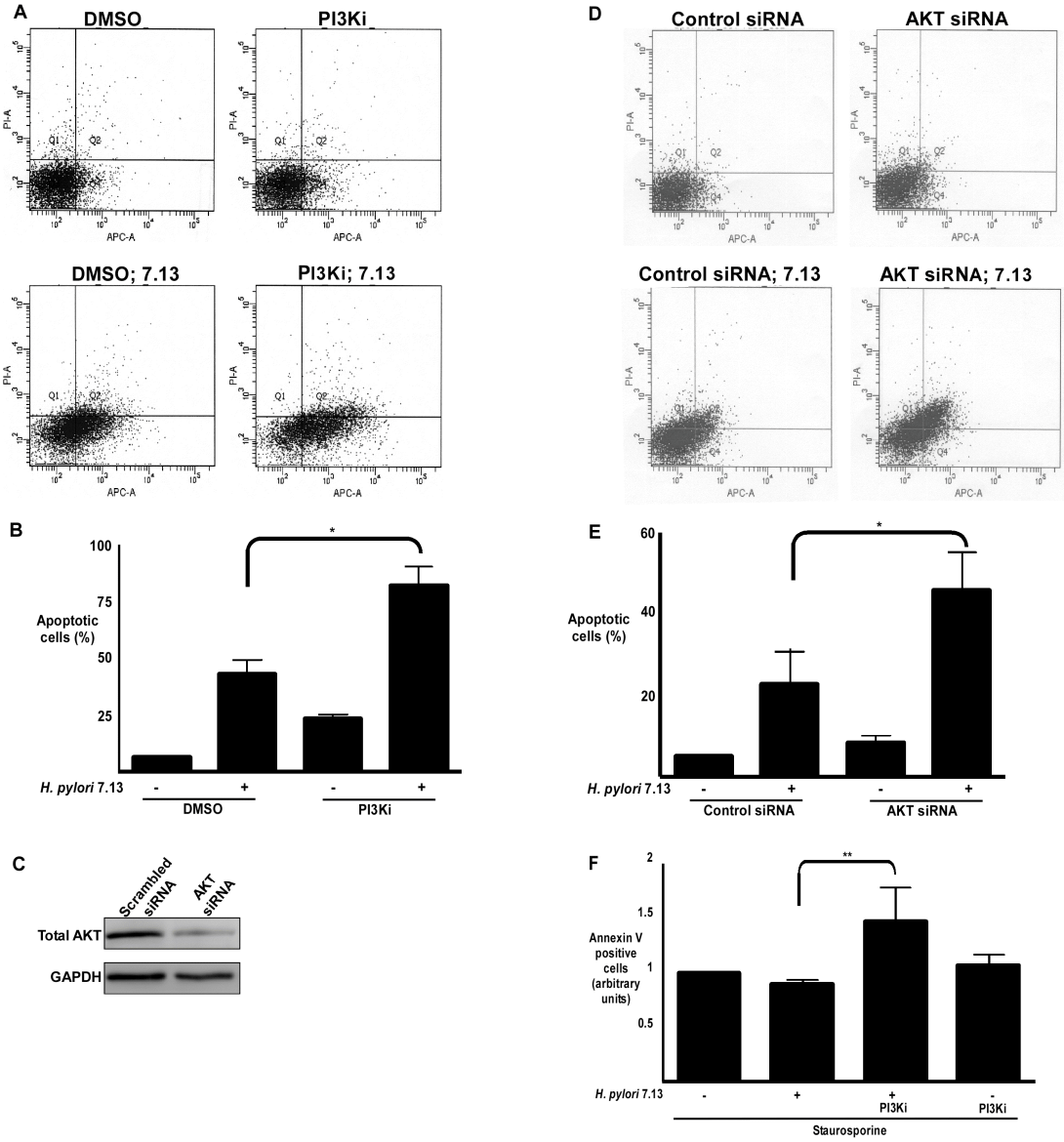

Figure 14. Activation of AKT by *H. pylori* promotes cell survival. ***(A)*** AGS cells were co-cultured with *H. pylori* strain 7.13 at a bacteria/cell concentration of 100:1, in the absence or presence of the PI3K inhibitor LY294002 (50 μM) or vehicle alone (DMSO) for 24 hours. Live cells were stained with Annexin V-APC and PI, and apoptosis was quantified by flow cytometry. The upper right quadrant represents late apoptosis, and the lower right quadrant represents early apoptosis. ***(B)*** Combined percentage of early and late apoptotic cells for experiments performed on at least 5 occasions. (-), cells incubated without *H. pylori*. Error bars = SEM. * $P < 0.005$ vs AGS cells infected with *H. pylori* strain 7.13 at MOI of 100:1 in the presence of vehicle alone. ***(C)*** AGS cells were transiently transfected with scrambled or AKT-specific siRNA, total protein was extracted and subjected to Western blot analysis using an anti-AKT antibody. ***(D)*** AGS cells transiently transfected with control or AKT-specific siRNA were co-cultured with

H. pylori strain 7.13 at a bacteria/cell concentration of 100:1 for 24 hours. Live cells were stained with Annexin V-APC and PI, and apoptosis was quantified by flow cytometry. The upper right quadrant represents late apoptosis, and the lower right quadrant represents early apoptosis. ***(E)*** Combined percentage of early and late apoptotic cells for experiments performed on at least 3 occasions. (-), cells incubated without *H. pylori*. Error bars = SEM. * $P < 0.05$ vs AKT siRNA-treated AGS cells infected with *H. pylori* strain 7.13. ***(F)*** AGS cells were co-cultured with or without *H. pylori* strain 7.13 at a bacterial/cell concentration of 100:1, treated with LY294002 (50μM) or medium alone, and then exposed to Staurosporine (Stsp). Cells were then stained with Annexin V-APC and PI, and subjected to flow cytometry. (-), cells incubated without *H. pylori*. ** $P<$ 0.05 vs AGS cells infected with *H. pylori* strain 7.13 in the presence of Stsp alone.

Discussion

PI3K is a host signaling molecule related to carcinogenesis. Our current experiments have shown that activation of PI3K-AKT can regulate microbially-induced carcinogenic responses by 1) demonstrating that *H. pylori* can induce phosphorylation and activate AKT in gastric epithelial cells *in vitro*, 2) capitalizing on an *H. pylori* isogenic mutant system to demonstrate a requirement for peptidoglycan translocation in AKT activation, 3) defining upstream signaling mediators of *H. pylori*-induced AKT activation and 4) combining transient inhibitor and gene silencing techniques with studies of epithelial responses that have carcinogenic potential (e.g. cell survival). Collectively, these studies indicate that *H. pylori* co-opts the PI3K-AKT signaling cascade, which, over prolonged periods of time, may lower the threshold for carcinogenesis.

In contrast to extensive literature invoking PI3K and AKT as tumorigenic molecules, few reports have examined the effects of bacterial pathogens on this signaling cascade. *Haemophilus influenzae* activates PI3K-AKT in epithelial cells, which then leads to a down-regulation of p38-MAPK activation [338]. *Salmonella* exploits PI3K in intestinal epithelial cells as an anti-inflammatory signal to reduce IL-8 production, which may contribute to the establishment of colonization in the intestine [102]. Our results suggest that induction of PI3K-AKT signaling by *H. pylori* requires a functional *cag* secretion apparatus and peptidoglycan, revealing a previously unrecognized effect of this *cag* island substrate, since the only defined role to date of *cag*-mediated peptidoglycan delivery is NOD1-dependent induction of IL-8 secretion [169, 228]. In other cell

systems, such as eosinophils, peptidoglycan has been shown to activate PI3K signaling and to regulate IL-8 production through Toll-like receptor (TLR) 2 [189]. However, further experiments are required to determine the precise mechanism through which PI3K is activated in *H. pylori*-infected gastric epithelial cells.

Hyperproliferation has been reproducibly demonstrated in *H. pylori*-infected tissue [5, 192] and this is accompanied by decreased levels of apoptosis in colonized human and rodent gastric epithelium [104]. Several reports have demonstrated that one role of AKT is to inhibit the function of caspases, which induce apoptosis and cell-cycle arrest. In addition to PI3K activation, however, *H. pylori* activates other pathways that influence cell survival. For example, MEK/ERK activation in response to *H. pylori* has been shown to increase Mcl-1 levels, leading to apoptosis resistance [81]. The collective result of activation of these pathways is inhibition of apoptosis and increased cell proliferation, events that favor tumorigenesis.

In summary, *H. pylori* induces PI3K-AKT signaling in gastric epithelial cells, which requires the *cag* secretion system and peptidoglycan as well as EGFR transactivation and Src activation in host cells. *H. pylori*-induced PI3K activation mediates protection from apoptosis, a phenotype related to carcinogenesis. Taken together, these data present insights into the pathogenic mechanisms underlying *H. pylori* infection.

CHAPTER III

CELL PROLIFERATION IN RESPONSE TO *HELICOBACTER PYLORI* IS STIMULATED BY PPARδ

Summary

Helicobacter pylori infects approximately half of the world's population and is the strongest known risk factor for the development of gastric cancer. One *H. pylori* virulence constituent that augments the risk for gastric injury is the *cag* secretion system, which translocates the bacterial effectors CagA and peptidoglycan (PGN) into host cells. Peroxisome proliferator-activated receptor δ (PPARδ) is a ligand-activated transcription factor and PPARδ signaling promotes tumor growth in models of gastrointestinal carcinogenesis. We defined the role of *H. pylori* virulence factors and activation of PPARδ in regulation of cell proliferation, a host response that contributes to carcinogenesis. Our experiments demonstrate that a carcinogenic *H. pylori* strain, 7.13, induces expression and activation of PPARδ. PPARδ activation by strain 7.13 stimulated epithelial cell proliferation, which may be mediated by the PPARδ target *cyclin E1*. These events are dependent upon structural components of the *cag* secretion system, and the *cag* substrates CagA and peptidoglycan. Because PPARδ regulates a multitude of host responses, activation of this molecule by *H. pylori* may contribute to varying levels of cellular turnover as well as the diverse pathologic outcomes associated with chronic *H. pylori* colonization.

Introduction

Helicobacter pylori-induced chronic gastritis significantly increases the risk for gastric adenocarcinoma, yet only a fraction of colonized persons ever develop neoplasia [221]. *H. pylori* strains isolated from different individuals are genetically diverse, and several microbial constituents have been identified that augment cancer risk [138]. The *cag* pathogenicity island is a strain-specific locus that encodes a type IV bacterial secretion system, and the product of the terminal gene in the island (CagA) is translocated into host epithelial cells and undergoes tyrosine phosphorylation at specific motifs by Src and Abl kinases [22, 128, 208, 269]. Phospho-CagA subsequently activates a eukaryotic phosphatase (SHP-2), leading to morphological changes that are reminiscent of unrestrained stimulation by growth factors [128]. Non-phosphorylated CagA also exerts effects within host cells that contribute to pathogenesis. We and others have demonstrated that translocation, but not phosphorylation, of CagA leads to aberrant activation of β-catenin, disruption of apical-junctional complexes, and a loss of cellular polarity, alterations that play a role in carcinogenesis [10, 99, 340]. In addition to CagA, the *cag* secretion system also delivers components of *H. pylori* peptidoglycan into host cells where they are recognized by Nod1, an intracytoplasmic pattern-recognition molecule [319]. Nod1 sensing of *H. pylori* peptidoglycan activates NF-κB, as well as the phosphatidylinositol 3-phosphate kinase (PI3K) signaling cascade, leading to pro-inflammatory cytokine release and β-catenin activation [199, 200, 297, 319].

Host molecules that may influence gastric carcinogenesis in conjunction with *H. pylori* include β-catenin and p120-catenin (p120). β-catenin is normally bound to E-cadherin at the cell membrane or sequestered in the cytosol within a multi-protein inhibitory complex that contains APC, GSK-3β and axin [308]. When Wnt signaling is inactive, β-catenin is constitutively phosphorylated by GSK-3β and targeted for proteosomal degradation [308]. Binding of Wnt to its receptor inhibits β-catenin degradation, leading to its nuclear accumulation and the formation of heterodimers with LEF/TCF transcription factors and targeted up-regulation of genes that influence carcinogenesis [308]. Within the context of *H. pylori* infection, CagA has been demonstrated to physically interact with E-cadherin leading to release of β-catenin into the cytoplasm and nucleus [159]. However, recent reports have demonstrated that *H. pylori* can activate β-catenin via PI3K-dependent inactivation of GSK-3β, although the specific bacterial factors required for these events remain unclear [200, 297].

p120 is a multifunctional host protein that localizes to the cell membrane to mediate cell-cell adhesion [244, 245]. p120 can also aberrantly localize to the nucleus where it binds Kaiso, a transcriptional repressor of β-catenin target genes [246, 290]. *H. pylori* induces nuclear translocation of p120 in gastric epithelial cells in a *cag*-dependent manner, which increases *mmp7* expression via relief of Kaiso-mediated transcriptional repression [210]. In addition to *mmp-7,* p120 and Kaiso also mediate expression of additional β-catenin target genes, including peroxisome proliferator-activated receptor δ (*pparδ*) [217]. PPARδ is a member of the nuclear hormone receptor superfamily, [324] and through heterodimer formation with the retinoid X receptor (RXR), PPARδ regulates transcription

of target genes that mediate fatty acid oxidation and glucose utilization [324]. Recent evidence, however, suggests that cross regulation between β-catenin signaling and PPARδ influences oncogenesis at other sites within the gastrointestinal tract [196].

Levels of PPARδ are elevated in most human colorectal cancers as well as carcinomas that develop in murine models of colon cancer and the PPARδ agonist GW501516 has been shown to be proneoplastic in mice [119, 120, 125]. Similarly, deletion of PPARδ decreases intestinal adenoma growth in $Apc^{Min/+}$ mice and inhibits the tumor promoting effects of GW501516 [324]. However, two studies reported that disruption of PPARδ increased polyp formation in $Apc^{Min/+}$ mice in the absence of exogenous PPARδ stimulation, which has necessitated in-depth studies [123, 240]. An independent study using a xenograft model revealed that disruption of both PPARδ alleles in human HCT-116 colon carcinoma cells decreased tumorigenicity, supporting the concept that activation of PPARδ promotes tumor growth [216]. Further, the PPARδ agonist GW501516 stimulates proliferation in human breast, prostate, and hepatocellular carcinoma cells, and, in a murine mammary tumor model, treatment with GW501516 accelerated tumor formation [349]. Consistent with these findings are recent observations that cyclin E1, a cell cycle regulatory protein that drives cells from G1 into S phase via hyperphosphorylation of the retinoblastoma protein Rb is a PPARδ target [351]. Since *H. pylori* can activate host effectors that regulate expression of PPARδ, the goal of this study was to define whether up-regulation of PPARδ mediates gastric epithelial responses with carcinogenic potential *in vitro* and *in vivo*.

Experimental Procedures

H. pylori strains

The *H. pylori cag*$^{+}$ rodent-adapted strain 7.13 was grown in *Brucella* broth with 5% FBS for 18 hours, harvested by centrifugation, and was added to gastric cells at a bacteria-to-cell ratio of 100:1. Isogenic *cagA*$^{-}$, *cagE*$^{-}$, and *slt*$^{-}$ null mutants were constructed within strain 7.13 by insertional mutagenesis using *aphA* and were selected with kanamycin (25 μg/ml) as described previously [223]. The *cagA*$^{-}$/*slt*$^{-}$ double mutant was constructed within strain 7.13 by insertional mutagenesis using *aphA* and the chloramphenicol (*cat*) resistance cassette from pBSC103, respectively, and was selected with kanamycin and chloramphenicol (10 μg/ml).

Cell Culture, Plasmids and Reagents

MKN28 human gastric epithelial cells were grown in RPMI medium 1640 (GIBCO/BRL) with 10% FBS (Sigma) and 20 μg/ml gentamicin (GIBCO/BRL) under 5% CO_2 at 37°C. The PI3K pharmacological inhibitor LY294002 (Cell Signaling Technology) was used at a concentration of 12.5 μM. The PPRE3-tk-luciferase reporter plasmid and dominant-negative PPARδ construct were kind gifts from D. Wang (Vanderbilt University). Topflash and Fopflash reporter plasmids were kind gifts from K. Kinzler and B. Vogelstein (Johns Hopkins University). The PPARδ-specific ligand, GW501516 (Cayman Chemical Co., Ann Arbor, MI) was used at a final concentration of 10 nM.

Viral production and retroviral transduction

Phoenix 293 packaging cell lines at 50% confluence were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Fresh medium was added 24 h after transfection, and tissue culture medium was collected and filtered through a 0.45-μm filter 72 h after transfection. For retroviral transduction, MKN28 cells at 50% confluence were incubated overnight with freshly harvested virus containing 4 μg/ml Polybrene (American Bioanalytical). To generate stable cell lines, cells transduced with the pSUPER.retro.puro virus were selected with 1.5 μg/ml puromycin for 48 h. Clonal populations were selected using cloning rings and limiting dilution techniques.

Transient transfection of siRNA.

MKN28 cells (4×10^5) in 12-well plates were transiently transfected using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Briefly, transfection reagent (2.0 μl/well) was mixed with siRNA oligos (5 μl of 20 μM solution/well) in 200 μl Opti-MEM (Life Technologies). Cells were incubated with the transfection mixture for 24 hours, fresh medium was added, and bacterial co-cultures were performed 24 hours later.

Western blot analysis

Cells were lysed in RIPA buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 1% Triton X-100, and 0.1% SDS) containing protease inhibitor cocktail, and protein concentrations were quantified by the Bradford assay. Proteins (30 μg) were separated by SDS-

polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes (PVDF, Pall, Ann Arbor, MI). Protein levels were assessed by Western blotting by using anti-PPARδ antibody (1:500, Santa Cruz Biotechnology), anti-β-catenin antibody (1:1000, Sigma Aldrich), anti-p120 antibody (1:1000, Abcam), or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:2000; Millipore Bioscience Research Reagents). Primary antibodies were detected using goat anti-mouse, goat anti-rabbit, or donkey anti-goat (1:5000; Santa Cruz Biotechnology) horseradish peroxidase-conjugated secondary antibodies and visualized by Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life and Analytical Sciences) according to the manufacturer's instructions on a Chemigenius system (Syngene). For cellular fractionation, cytoplasmic and nuclear fractions were obtained using the Q-Proteome Cell Compartment Kit (Qiagen).

Primary Gastric Cell Extraction and Culture

All animal studies were approved by the Vanderbilt Institutional Animal Care and Usage Committee. Stomachs were removed from 8-wk-old male wild-type C57Bl/6 and PPARδ$^{-/-}$ C57Bl/6 mice (provided by D. Wang, Vanderbilt University), ligated at the pylorus and esophagus, inverted, and injected with 1 ml of 0.5 mg/ml collagenase A as described previously [339]. Stomachs were then washed in Hanks' balanced salt solution (HBSS) three times at 37°C. Tissue was incubated in 10 ml of 1 mM dithiothreitol for 15 minutes at 37°C with shaking, washed in HBSS three times at 37°C, and incubated in 0.37 mg/ml collagenase for 30 minutes at 37°C. After the first collagenase digestion, samples were washed again in HBSS (3 times at 37°C) and incubated for a further 30

minutes in collagenase (0.37 mg/ml; 37°C). Tissue was triturated using a wide-mouthed pipette, and larger fragments of tissue were allowed to settle under gravity for 45 seconds. The supernatant containing isolated gastric cell colonies was removed and transferred to a clean 50-ml conical tube, shaken vigorously to release additional colonies, and left on ice to sediment for 30 minutes. The supernatant was then carefully removed and discarded, and isolated cell colonies were plated on chamber slides. Colonies of gastric epithelial cells were cultured in DMEM NUT Mix F-12 (Ham's) supplemented with 10% FBS and 1% antibiotic-antimycotic solution. Colonies were then incubated in a humidified incubator at 37°C under an atmosphere of 5% CO_2.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

MKN28 cells were grown to confluence and then co-cultured with *H. pylori* or medium alone for 6, 12, 24 and 48 hours. RNA was prepared from co-culture lysates using RNAeasy kit (Qiagen) following the manufacturer's instructions. Reverse transcriptase-PCR was performed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA), which was followed by real-time quantitative PCR using SYBR green (Applied Biosystems, Foster City, CA) and the 7300 real-time PCR system (Applied Biosystems, Foster City, CA). The relative differences between treatment groups were calculated based on values for the gene of interest normalized to values of the hypoxanthine phosphoribosyltransferase 1 gene (*hprt1)*. The primers used were:

pparδ, forward 5' GAGGAAGTGGCCACGGGTGAC 3' and

reverse 5' CCACCTGAGGCCCCATCACAG 3';

cyclin E1, forward 5' TCATTTACAGCCTTGGGACAA 3' and

reverse 5' AGCGAACAGGAAGACTCAAGC 3';

hprt, forward 5' TTGGAAAGGGTGTTTATTCCTCA 3' and

reverse 5' TCCAGCAGGTCAGCAAAGAA 3'.

PPARδ and β-catenin transcriptional assays

MKN28 cells (2 x 10^5) plated in 12-well plates were transiently transfected with 4 µl Lipofectamine 2000 (Invitrogen), 0.3 µg PPRE-tk-luciferase/ 5 ng of pRL-SV40, and 0.4 µg empty vector or dominant-negative PPARδ for 5 hours. Twenty-four hours post-transfection, cells were co-cultured with *H. pylori* and then harvested in 1x Passive Lysis Buffer (Promega). For β-catenin studies, MKN28 cells (2 x 10^5) were transfected with 4 µl Lipofectamine 2000, 1 µg/ml Topflash or 1 µg/ml Fopflash, and 5 ng pRL-SV40 in Opti-MEM (Life Technologies) for 5 hours. Transfection mixtures were then replaced with complete medium containing *H. pylori* or medium alone. After 24 hours, cells were harvested in 1x Passive Lysis Buffer (Promega). Luciferase activity was determined using a luminometer and normalized to *Renilla* luciferase using the Dual-Luciferase assay kit (Promega).

Three-dimensional Matrigel proliferation assay

Forty-eight-well tissue culture plates were coated with 100 µl of thawed BD Matrigel™ Basement Membrane Matrix and were placed at 37°C for 30 minutes for Matrigel solidification. MKN28 cells (2 x 10^4) transfected with empty vector or dominant-negative PPARδ were overlayed on Matrigel™ and, after 24 hours, cells were infected with *H. pylori,* medium alone and/or GW501516. Cells were removed every 24 hours

from Matrigel™ using BD Cell Recovery Solution according to the manufacturer's protocol and enumerated using Trypan Blue or BrdU incorporation (Roche).

Immunofluorescence

Primary gastric colonies were cultured in glass chamber slides and were co-cultured with *H. pylori* or medium alone for 48 hours. Cells were then washed twice with Dulbecco's phosphate-buffered saline (DPBS) and fixed with 4% paraformaldehyde in DPBS for 10 minutes. Cells were rinsed with DPBS and subsequently permeabilized for 30 minutes with DPBS containing 0.1% Triton X-100, followed by incubation in 3% BSA for 1 hour at room temperature. Slides were immunostained with goat monoclonal anti-PPARδ antibody (Santa Cruz), rabbit polyclonal anti-Cyclin E antibody (Santa Cruz) or rabbit anti-*H. pylori* antibody (Dako) at a concentration of 1:100, overnight at 4°C. Washed slides were incubated with goat anti-rabbit AlexaFluor 488-conjugated antibody (Invitrogen) respectively, at a concentration of 1:100 for 1 hour at room temperature. Slides were washed and incubated with TO-PRO dimeric cyanine nucleic acid dye at a concentration of 1:100 for 20 minutes at room temperature (Invitrogen). Slides were then mounted using ProLong Gold antifade reagent (Invitrogen). Imaging was performed on an LSM 510 confocal microscope (Carl Zeiss, Thornwood, NY) by using 40x /1.30 Plan-NeoFluar oil objective at room temperature, and acquisition was performed using the manufacturer's proprietary software.

Statistical Analysis.

All *in vitro* experiments were performed on at least three independent occasions. Statistical analysis was performed by Student's t test and ANOVA using Prism Graph Pad. A P-value < 0.05 was defined as statistically significant.

Results

PPARδ is expressed and is functionally active in gastric epithelial cells.

To determine whether *H. pylori* induces PPARδ expression, MKN28 gastric epithelial cells were co-cultured with the wild-type *cag*$^{+}$ strain 7.13 and real time reverse transcription-PCR was performed. PPARδ mRNA expression was significantly increased in cells infected with *H. pylori,* beginning at 24 hours post-infection (**Figure 15A**), and this was accompanied by increased protein levels of PPARδ in both the cytosol and nucleus by 48 hours post-infection (**Figure 15B**).

To determine whether endogenous PPARδ was functionally responsive to *H. pylori*, MKN28 cells were transfected with a reporter vector (PPRE3-tk-luc) containing three tandem repeats of the PPAR response element (PPRE) from the acyl-CoA oxidase gene upstream of a firefly luciferase cDNA. As expected, treatment of transfected cells with the PPARδ selective agonist GW501516 increased luciferase activity (**Figure 15C**). To determine the effect of *H. pylori* on PPARδ activation, PPRE-transfected cells were infected with strain 7.13. Luciferase activity was significantly increased in infected compared to uninfected cells (**Figure 15C**).

To confirm the specificity of this response, MKN28 cells were co-transfected with PPRE and dominant-negative PPARδ (dnPPARδ). The dnPPARδ construct contains an inactivating mutation within the AF-2 domain, and retains its DNA-binding domain but lacks the ability to recruit transcriptional co-activators, thereby antagonizing PPARδ

signaling. PPARδ activity in response to *H. pylori* was abolished in cells transfected with dnPPARδ, indicating that activation of PPARδ by *H. pylori* is specific (**Figure 15C**). Thus, PPARδ is induced and functionally active in gastric epithelial cells infected with *H. pylori*.

p120 is required for H. pylori-mediated up-regulation of PPARδ expression.

PPARδ is a transcriptional target of β-catenin signaling, and Kaiso and p120 regulate expression of β-catenin target genes. Therefore, we investigated the relationship between β-catenin and PPARδ by examining levels of PPARδ in *H. pylori*-infected MKN28 cells treated with β-catenin-specific siRNA. Densitometric analysis of Western blots revealed an approximate 70% reduction in β-catenin protein levels with siRNA treatment (**Figure 16A**). PPARδ mRNA (data not shown) and protein expression in response to *H. pylori* was significantly decreased in cells with attenuated β-catenin compared to infected control cells (**Figure 16B**).

Since p120 can regulate expression of β-catenin target genes, we next determined if PPARδ is regulated by p120. MKN28 cells stably transduced with control or p120-specific siRNA (**Figure 16C**) were co-cultured with *H. pylori* strain 7.13 or medium alone. As expected, *H. pylori* significantly increased levels of *pparδ* mRNA by 24 hours in control MKN28 cells. However, the observed increase in *pparδ* expression was attenuated in *H. pylori*-infected p120 deficient cells when compared to infected controls, indicating that p120 is also required for *H. pylori*-mediated transcriptional up-regulation of PPARδ (**Figure 16D**).

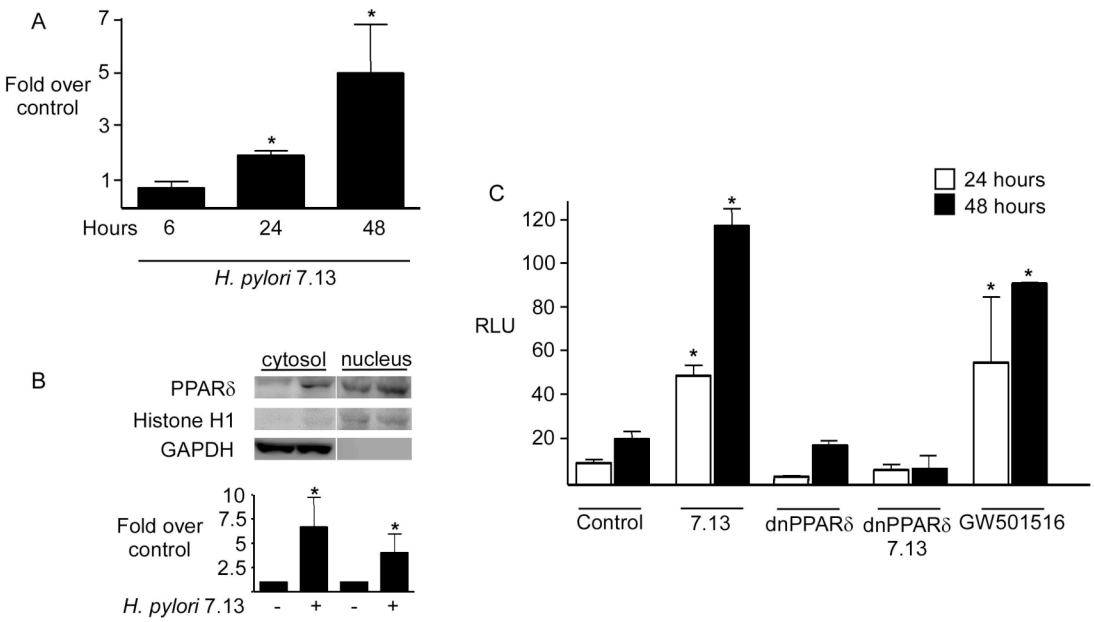

Figure 15. *Helicobacter pylori* induces expression and functional activation of PPARδ in gastric epithelial cells. ***(A)*** MKN28 cells were co-cultured with the *H. pylori* *cag*$^{+}$ strain 7.13 (MOI = 100) or medium alone. At defined time points, RNA was extracted, subjected to reverse transcription, and analyzed in duplicate by real-time PCR. Data represent –fold induction of *pparδ* mRNA in cells co-cultured with *H. pylori* versus medium alone from experiments performed on at least three occasions. Error bars, SEM. *$p < 0.05$ versus medium alone. ***(B)*** MKN28 cells were co-cultured with *H. pylori* strain 7.13 (MOI = 100). Forty-eight hours post-infection, total protein was extracted, subjected to subcellular fractionation, and analyzed by Western blot using an anti-PPARδ antibody. Representative blots are shown. Anti-Histone H1, and anti-GAPDH antibodies served as normalization controls for purification of nuclear and cytosolic subcellular fractionations, respectively. Densitometric analysis of multiple Western blots performed on at least three occasions. Graph represents fold PPARδ expression in infected versus uninfected cells. Error bars, SEM. *$p < 0.05$. ***(C)*** MKN28 cells were co-transfected with $PPRE_3$-tk-luciferase and pRL-SV40 with empty vector or dominant-negative PPARδ plasmids followed by treatment with *H. pylori* strain 7.13 or the PPARδ agonist GW501516. Dual luciferase assays were performed as described in Experimental Procedures. Error bars, SEM. *$p < 0.0004$.

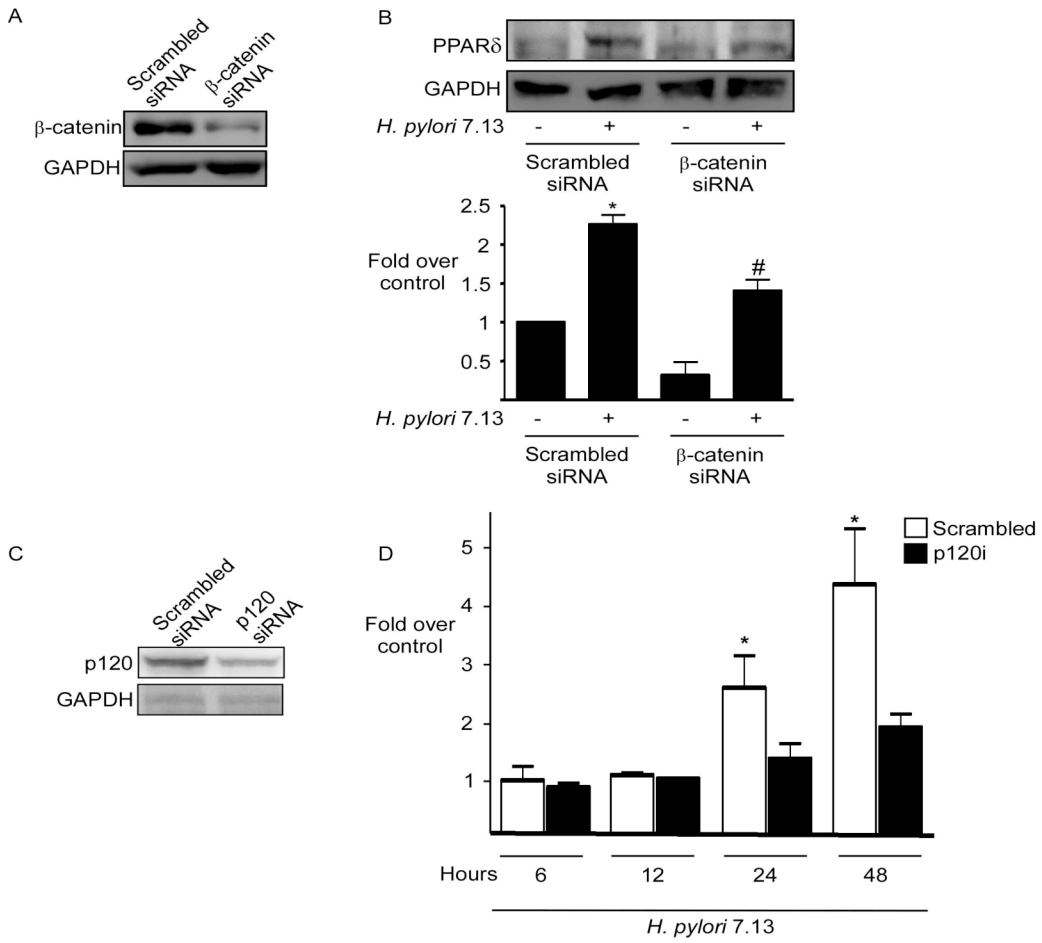

Figure 16. PPARδ expression is induced by *H. pylori* and requires β-catenin and p120 signaling. ***(A)*** MKN28 cells were transiently transfected with scrambled or β-catenin-specific siRNA; total protein was extracted and subjected to Western blot analysis using an anti-β-catenin antibody. ***(B)*** MKN28 cells transiently transfected with control or β-catenin- specific siRNA were co-cultured with *H. pylori* strain 7.13 (MOI = 100) for 24 hours. Total protein was extracted and analyzed by Western blot using an anti-PPARδ antibody. Representative blots are shown. Anti-GAPDH antibody served as normalization control. Bar graph represents densitometric analysis of multiple experiments. Data are represented as fold induction of PPARδ expression in infected versus uninfected. Error bars, SEM. *$p < 0.05$ versus medium alone. #$p < 0.05$ versus 7.13. ***(C)*** MKN28 cells were retrovirally transduced with either scrambled or human p120-specific siRNA (p120i), and clonal populations were selected. Total protein was extracted from control or p120i cells and analyzed by Western blot using a monoclonal anti-p120 antibody. GAPDH Western blots served as normalization controls. ***(D)*** Scramble control or p120i cells were co-cultured with *H. pylori* strain 7.13 (MOI = 100) or medium alone. At defined time points, total RNA was extracted, subjected to reverse transcription, and analyzed in duplicate by real-time PCR. Data are represented as fold induction of *pparδ* expression in infected versus uninfected cells for experiments performed on at least three occasions. Error bars, SEM. *$p < 0.05$ versus medium alone.

cag island effectors are required for PPARδ activation.

The *H. pylori cag* pathogenicity island induces epithelial responses that may lower the threshold for gastric cancer; therefore, we next directly examined the effects of *cagA*, *cagE*, and the *cag* island substrate peptidoglycan on PPARδ activation. To examine the role of peptidoglycan, we generated an isogenic *H. pylori* mutant lacking a critical enzyme required for peptidoglycan synthesis, soluble lytic transglycosylase (*slt*).

MKN28 cells transfected with the PPARδ responsive reporter were infected with wild-type strain 7.13 or its isogenic *cagA*$^-$, *cagE*$^-$, or *slt*$^-$ null mutant derivatives as well as a *cagA*$^-$/*slt*$^-$ double mutant strain. Loss of *cagA* or *slt* alone led to partial attenuation of PPARδ activation compared to levels induced by the wild-type strain (**Figure 17A**). However, inactivation of the *cag* secretion system structural component *cagE,* or *cagA* and *slt* in combination further attenuated the ability of strain 7.13 to activate PPARδ.

These results suggested that PPARδ activation in response to *H. pylori* may require two independent pathways: one that is regulated by translocation of CagA and the other which regulates induction of PI3K by peptidoglycan. To examine this more definitively, we measured PPARδ activity in response to *H. pylori* strain 7.13 in the presence or absence of the PI3K inhibitor LY294002, in order to substitute for loss of peptidoglycan. Compared to levels induced by wild-type *H. pylori*, PPARδ activation was significantly reduced in the presence of PI3K inhibition, similar to levels induced by infection with the *slt* null mutant (**Figure 17B**). To inactivate CagA- and peptidoglycan-dependent pathways in tandem, cells were pretreated with the PI3K inhibitor and then infected with

the 7.13 isogenic *cagA*$^-$ mutant. In cells dual-treated, luciferase activity was reduced to levels observed in uninfected control cells and was significantly decreased when compared to cells infected with wild-type 7.13 in the presence of PI3K inhibition alone (**Figure 17B**). These results indicate that induction of PPARδ requires both translocation of CagA and activation of PI3K signaling by *H. pylori* peptidoglycan.

Activation of β-catenin requires CagA and peptidoglycan.

β-catenin regulates the expression of PPARδ in intestinal epithelial cells; therefore we next determined if the microbial constituents required for *H. pylori*-induced PPARδ activation also mediated β-catenin activation in gastric epithelial cells. As expected, infection of MKN28 cells with strain 7.13 induced translocation of β-catenin into the nucleus (**Figure 17C**).

We next examined the role of translocated *cag* effector molecules on β-catenin activation in this system. MKN28 cells were transfected with a reporter construct containing 3 tandem LEF/TCF binding motifs upstream of the luciferase gene (Topflash) or a control construct containing mutated LEF/TCF sites (Fopflash), and then infected with *H. pylori* wild-type strain 7.13 or its isogenic mutants. Luciferase activity did not differ in cells transfected with the control construct with or without *H. pylori* (data not shown); however, activity was significantly higher in *H. pylori*-infected versus uninfected cells harboring the β-catenin responsive LEF/TCF construct, indicating that β-catenin is functionally responsive to *H. pylori* strain 7.13 in MKN28 gastric epithelial cells (**Figure 17D**). Inactivation of *cagE* significantly reduced the ability of strain 7.13 to activate β-

catenin to levels observed in uninfected control cells (**Figure 17D**). Inactivation of *cagA* or *slt* alone partially attenuated the increase in β-catenin activation induced by the wild-type 7.13 strain, but not to levels observed in control cells. To determine if these two *cag* island substrates exerted synergistic effects on β-catenin activation similar to results observed for PPARδ activation (**Figure 17A**), cells were infected with the 7.13 *cagA*$^{-}$/*slt*$^{-}$ double mutant. Loss of both CagA and Slt completely abolished β-catenin activation as compared to reductions induced by loss of either constituent alone (**Figure 17D**). These findings indicate the multiple *H. pylori* constituents can mediate β-catenin and are concordant with our results focused on *H. pylori*-induced PPARδ activation (**Figure 17A**).

Activation of PPARδ by H. pylori promotes cell proliferation.

H. pylori is associated with increased gastric epithelial cell proliferation in colonized human and rodent gastric mucosa, and PPARδ stimulates proliferation of human carcinoma cells; therefore, we next examined the role of PPARδ in *H. pylori*-induced cellular proliferation. Proliferation was measured using a three-dimensional model system, which more accurately reflects the *in vivo* tissue microenvironment by providing interactions between cells, growth factors, and an extracellular matrix (**Figure 18A**). As expected, cells treated with GW501516 proliferated at a significantly higher rate than untreated cells (data not shown).

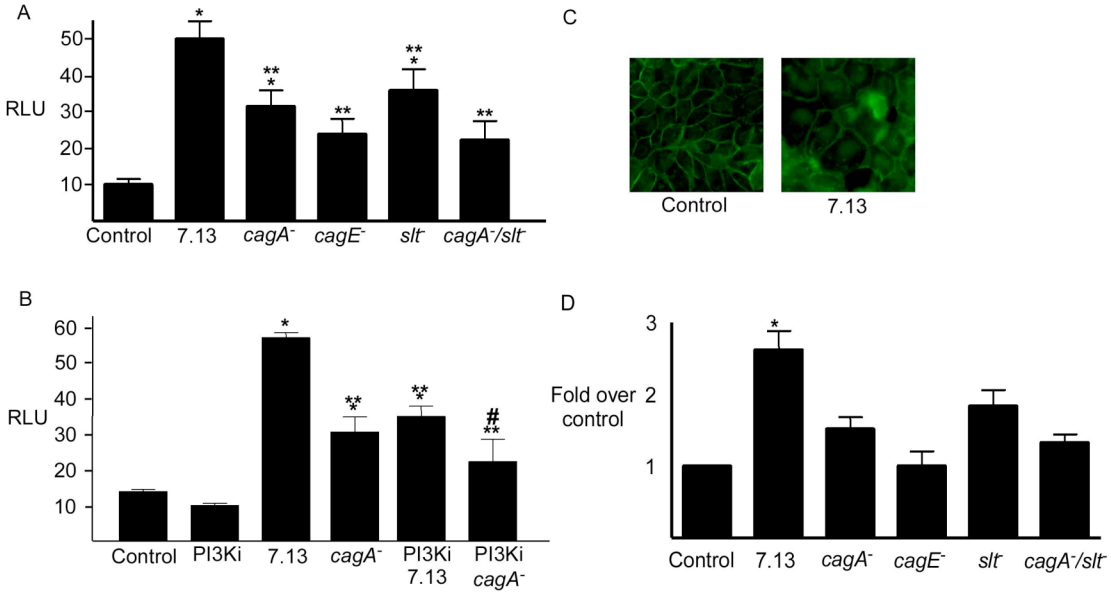

Figure 17. ***H. pylori*-induced PPARδ requires CagA and peptidoglycan.** ***(A)*** MKN28 cells were transiently transfected with $PPRE_3$-tk-luciferase and pRL-SV40 followed by infection with *H. pylori* strain 7.13 (MOI = 100), or the *cagA-, cagE-, slt- or cagA-/slt-* isogenic mutants. Dual luciferase assays were performed as described in Experimental Procedures. Error bars, SEM. *p < 0.05 versus Control. **p < 0.05 versus 7.13. ***(B)*** MKN28 cells were transiently transfected with $PPRE_3$-tk-luciferase and pRL-SV40, pre-treated with the PI3K inhibitor LY294002 (PI3Ki) for one hour, and infected with *H. pylori* strain 7.13 (MOI = 100), or its *cagA-* isogenic mutant. Dual luciferase assays were performed as described in Experimental Procedures. Error bars, SEM. *p < 0.05 versus Control. **p < 0.001 versus 7.13. #p < 0.01 versus PI3K; 7.13. ***(C)*** MKN28 cells were co-cultured with *H. pylori* strain 7.13 (MOI=100) or medium alone for 24 hours. Cells were stained with anti-β-catenin and AlexaFluor -488 antibodies and nuclear dye and analyzed by immunofluorescent microscopy. (400X). ***(D)*** MKN28 cells were transiently transfected with reporter constructs containing LEF-TCF binding motifs in the absence or presence of wild-type strain 7.13 or mutants lacking *cagA, cagE, slt* and/or *cagA*/*slt*. Luciferase activity was determined 24 h after infection. Error bars = SEM for experiments performed on at least 3 occasions. *p < 0.05 versus control.

After 72 hours of co-culture, treatment with *H. pylori* strain 7.13 significantly increased cell number compared to uninfected cells (**Figure 18B**). In contrast, cell number in *H. pylori*-infected cells expressing dnPPARδ was no different than uninfected controls.

These results were confirmed using an independent proliferation assay that measures incorporation of Bromodeoxyuridine (BrdU) during DNA synthesis. MKN28 cells were treated with scrambled or PPARδ-specific siRNA (**Figure 18C**) and were then seeded into Matrigel. After 96 hours of co-culture with *H. pylori*, cells were incubated with BrdU and quantified by ELISA. Absorbance values for *H. pylori*-infected control cells were significantly higher than uninfected cells or *H. pylori*-infected cells treated with PPARδ siRNA (**Figure 18D**), confirming that activation of PPARδ by *H. pylori* strain 7.13 promotes cellular proliferation in a model that incorporates important elements present within gastric mucosa.

H. pylori-induced expression of the cell-cycle regulator Cyclin E1 is PPARδ-dependent

The PPARδ target *cyclin E1* promotes cellular proliferation in a variety of model systems. Having demonstrated that PPARδ regulates *H. pylori*-induced proliferation, we next determined if *H. pylori* could induce expression of *cyclin E1* in a PPARδ-dependent manner.

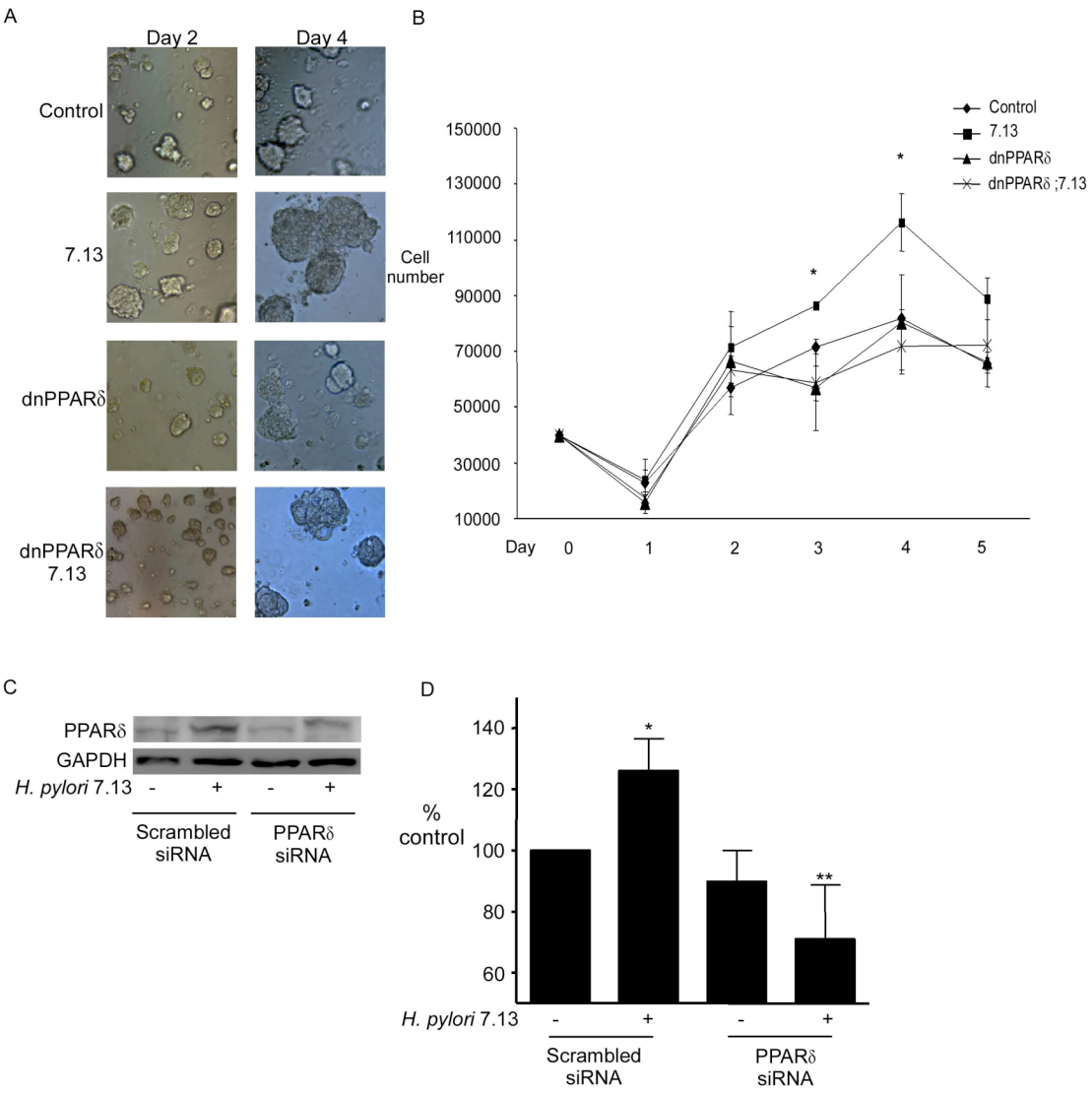

Figure 18. *H. pylori* infection stimulates proliferation of MKN28 cells in a PPARδ-dependent manner. ***(A)*** MKN28 cells were transfected with control vector or dominant-negative PPARδ constructs, were then seeded in a three-dimensional culture system and infected with strain 7.13 or medium alone. Representative images are shown. ***(B)*** Following infection with wild-type *H. pylori* strain 7.13, cells were removed from Matrigel at 24 hour intervals and enumerated using Trypan blue staining. Error bars = SEM for experiments performed on at least 3 occasions. *$p < 0.05$ versus control. ***(C)*** MKN28 cells transfected with scrambled or PPARδ-specific siRNA were co-cultured with *H. pylori* strain 7.13 for 24 hours. Total protein was extracted and analyzed by Western blot using an anti-PPARδ antibody. Representative blots are shown. Anti-GAPDH antibody served as normalization control. ***(D)*** MKN28 cells transfected with scrambled or PPARδ-specific siRNA were seeded in Matrigel and infected with *H. pylori* strain 7.13 for 96 hours. BrdU was added to culture medium and ELISA for BrdU incorporation was performed. Error bars = SEM for experiments performed on at least 3 occasions. *$p < 0.05$ versus control. **$p < 0.05$ vs 7.13-infected scrambled control.

MKN28 cells were transfected with scrambled or PPARδ-specific siRNA and then co-cultured with *H. pylori* or medium alone for 24 hours; siRNA reduced PPARδ levels by approximately 50% (**Figure 18C**). Reduction of PPARδ levels significantly decreased expression levels of *cyclin E1* in *H. pylori*-infected control cells (**Figure 19**), commensurate with the level of PPARδ knockdown, indicating that hyperproliferation that develops in response to *H. pylori* infection may be mediated by the PPARδ target *cyclin E1*.

H. pylori induces aberrant localization of PPARδ to the nucleus in ex vivo gastric cell colonies

Our current data demonstrate that *H. pylori* induces increased expression and activation of PPARδ in isolated human gastric epithelial cells. To extend these results, we capitalized upon a model of *H. pylori* infection that closely recapitulates cellular organization in the stomach. Gastric cell colonies were isolated from 8-week-old male C57Bl/6 mice, co-cultured with *H. pylori* strain 7.13 or medium alone for 48 hours, and PPARδ localization was assessed by laser scanning immunofluorescent microscopy. Consistent with our *in vitro* results, cytoplasmic accumulation and nuclear translocation of PPARδ was observed in cells co-cultured with *H. pylori*, but not in cells incubated with medium alone (**Figure 20A**).

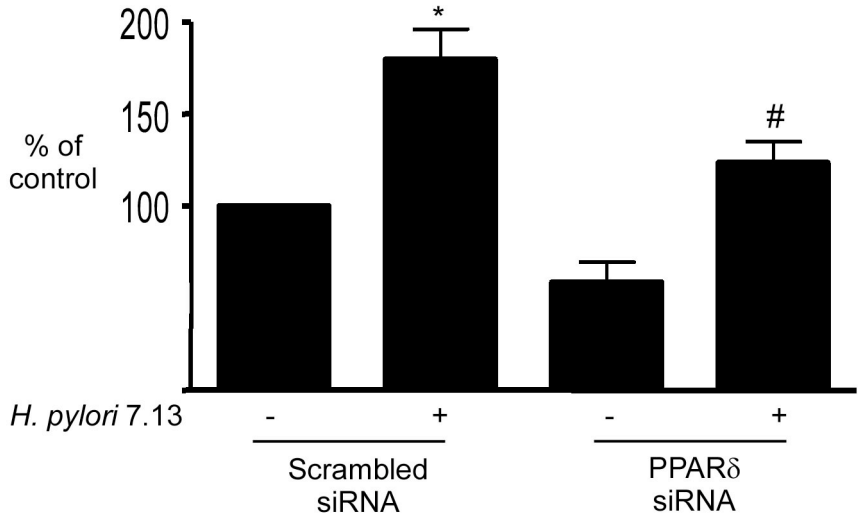

Figure 19. ***cyclin E1*** **upregulation by** ***H. pylori*** **requires PPARδ.** MKN28 cells transiently transfected with scrambled or PPARδ-specific siRNA were cocultured with *H. pylori* strain 7.13 (MOI = 100) or medium alone for 24 hours. RNA was extracted, subjected to reverse transcription, and analyzed in duplicate by real-time PCR. Data represented as % of control where uninfected, scrambled siRNA-transfected is 100%. Error bars, SEM. *$p < 0.05$ versus medium alone. #$p < 0.05$ versus 7.13.

To determine the relationship between PPARδ and Cyclin E1 in a genetic model of PPARδ deficiency, we also examined Cyclin E1 staining in glands obtained from wild-type or PPARδ$^{-/-}$ C57Bl/6 mice. *H. pylori* infection resulted in increased Cyclin E1 levels in wild-type gland colonies but not glands isolated from PPARδ$^{-/-}$ mice which remained similar to uninfected control cells (**Figure 20B**). These results indicate that, similar to cultured gastric epithelial cells, *H. pylori* can induce PPARδ and Cyclin E1 in a unique model system that mirrors events within colonized gastric mucosa.

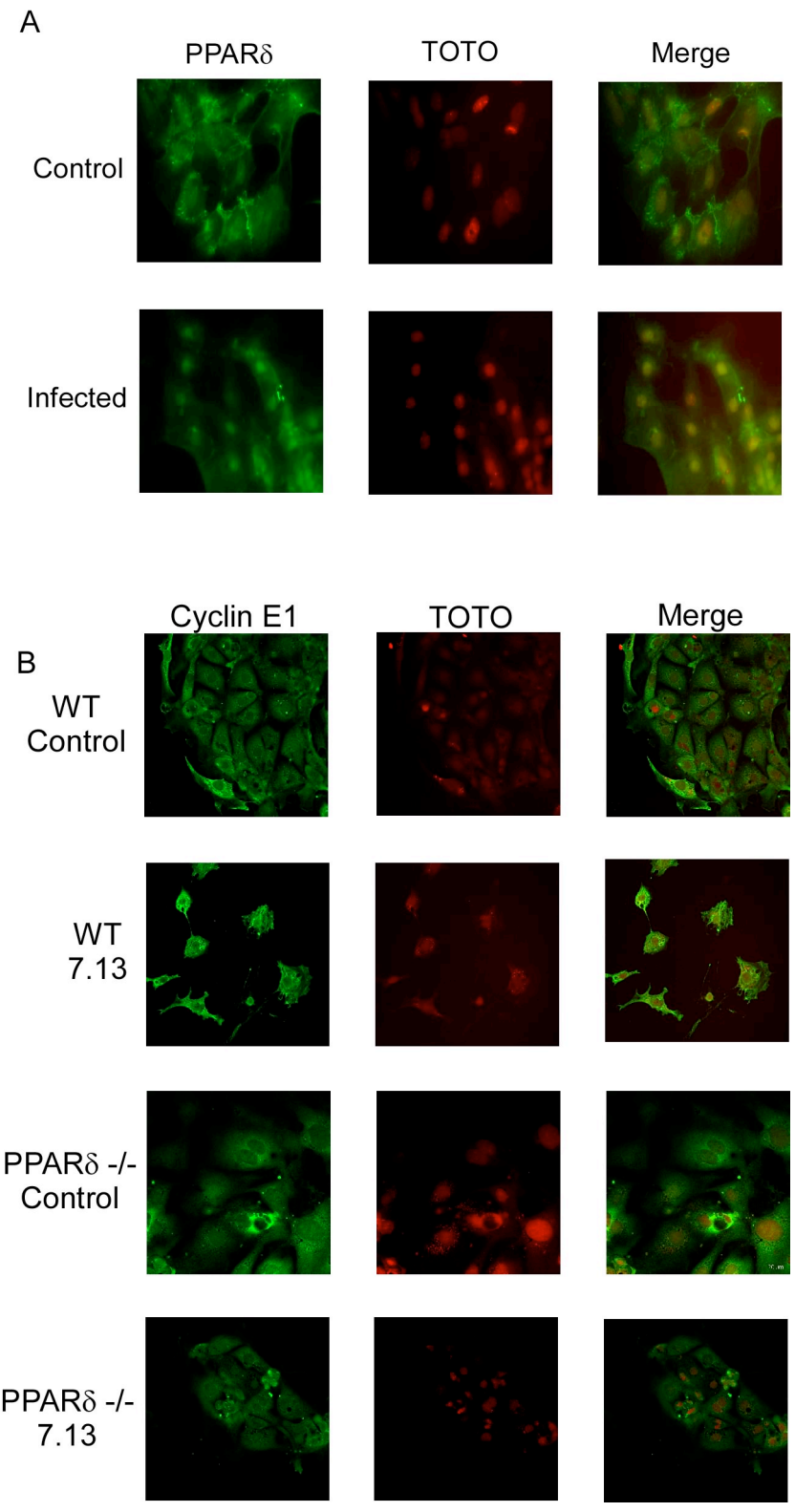
A
PPARδ
TOTO
Merge
Control
Infected
B
Cyclin E1
TOTO
Merge
WT
Control
WT
7.13
PPARδ -/-
Control
PPARδ -/-
7.13

Figure 20. *H. pylori* induces aberrant localization of PPARδ to the nucleus in *ex vivo* gastric colonies. ***(A)*** Primary murine gastric epithelial cell colonies were co-cultured with *H. pylori* strain 7.13 or medium alone for 24 hours. Cells were fixed and incubated with an anti-PPARδ antibody, followed by incubation with an anti-goat AlexaFluor-488 antibody and TO-PRO nucleic acid dye. Cells were visualized by laser scanning immunofluorescent microscopy. PPARδ, green; nuclei, red. 400x magnification. ***(B)*** Primary murine gastric epithelial cell colonies from wild-type C57Bl/6 or PPARδ$^{-/-}$ C57Bl/6 mice were co-cultured with *H. pylori* strain 7.13 or medium alone for 24 hours. Cells were fixed and incubated with an anti-cyclin E antibody, followed by incubation with an anti-goat AlexaFluor-488 antibody and TO-PRO nucleic acid dye. Cells were visualized by laser scanning immunofluorescent microscopy. Cyclin E, green; nuclei, red. 400x magnification.

Discussion

Our current experiments have identified PPARδ as a regulator of *H. pylori*-induced responses with carcinogenic potential by 1) demonstrating that *H. pylori* infection increases the expression and activation of PPARδ, 2) using a gastric cell model to demonstrate the requirement of p120 in transcriptional upregulation of PPARδ, 3) capitalizing on a three-dimensional culture system to show *H. pylori* stimulates epithelial cell proliferation, which may be mediated by the PPARδ target, cyclin E1, and 4) demonstrating *H. pylori* can alter the topography of PPARδ localization within a physiologically relevant *ex vivo* primary gland culture system. Collectively, these studies indicate that *H. pylori* activates PPARδ to regulate cell proliferation, a phenotype related to carcinogenesis.

Nuclear p120 relieves transcriptional repression exerted by Kaiso on β-catenin target genes that possess oncogenic properties [71, 217, 236]. Using stable knockdown of p120 in MKN28 gastric epithelial cells, we have now identified *pparδ* as a specific target of p120 signaling that may influence carcinogenesis within the context of *H. pylori* infection. The role of PPARs in fatty acid utilization and oxidation has been well established, but their functions in tumorigenesis have not been as clearly defined. In particular, PPARδ is the least characterized isoform of the PPAR family, although it presumably performs critical functions in embryonic development, fatty acid metabolism, wound healing and control of inflammation [27, 165, 185, 230, 302, 327]. Additional evidence now suggests PPARδ can influence colorectal cancer development as PPARδ

expression is elevated in most colorectal cancers and PPARδ$^{-/-}$ cells exhibit a decreased ability to form tumors in a xenograft model [119, 240]. We have now extended these observations into the gastric niche by demonstrating that a carcinogenic *H. pylori* strain induces PPARδ expression *in vitro* and *ex vivo*, which may contribute to *H. pylori*-mediated carcinogenesis.

Using a three-dimensional culture system that more closely reflects the *in vivo* microenvironment, we have shown that *H. pylori* promotes epithelial cell proliferation in a PPARδ-dependent manner. These findings are consistent with previous reports demonstrating the ability of PPARδ to stimulate proliferation in mouse adipocytes, breast, prostate and colon carcinoma cells [55, 121, 141, 229, 324, 343]. *In vitro* studies examining cell turnover in response to *H. pylori* has provided variable results, demonstrating both increased and decreased cell proliferation in response to infection [46, 89, 152, 214, 249, 278]. These discrepancies may be attributed to differences in bacterial strains, gastric cell lines and/or methods used to determine effects of *H. pylori* on proliferation. *In vivo,* however, chronic *H. pylori* infection induces epithelial hyperproliferation that is not balanced by increased levels of apoptosis [227]. Maintenance of tissue integrity requires that enhanced cell production be accompanied by increased rates of cell loss. Thus, hyperproliferation that is not balanced by a concordant increase in apoptosis, over long periods of infection, may heighten the risk for gastric cancer associated with *H. pylori cag*$^{+}$ strains. In this study we demonstrated that PPARδ activation by *H. pylori* requires a functional *cag* secretion system and the *cag* effectors

CagA and peptidoglycan, consistent with findings indicating that *cag*$^{+}$ strains more robustly alter rates of epithelial cell turnover.

We have also demonstrated that *H. pylori* induces *cyclin E1* expression in a PPARδ-dependent manner. Cyclin E1 regulates the rate limiting step in the transition from G1 to S phase of the cell cycle by hyperphosphorylation of the Rb protein, which is essential for DNA synthesis [109]. Cyclin E1 deregulation is a common event in oncogenesis [266], and the majority of gastric tumors have increased Cyclin E1 expression, with higher expression correlating with tumor invasion, lymph node metastasis and poor prognosis [2, 26, 347, 348]. Studies examining levels of Cyclin E1 in response to *H. pylori* infection have provided variable results, showing both upregulation, as well as inhibition of Cyclin E1 through increased expression of its inhibitor, $p27^{KIP1}$ [79, 288, 346]. Similar to studies revealing discrepancies in rates of cell turnover in response to *H. pylori,* expression of certain host cell proteins is likely to be variably affected by strain-specific and cell-specific factors. Ultimately, expression of Cyclin E1 in systems that closely reflect *H. pylori* infection *in vivo* will allow delineation of the importance of Cyclin E1 expression in gastric carcinogenesis. In this study, we demonstrated that Cyclin E1 is expressed in response to *H. pylori* infection of primary gastric glands that not only contain epithelial cells but also stromal and lamina propria cells. Consistent with our *ex vivo* studies, Yao *et al.* report overexpression of Cyclin E1 in Mongolian gerbils infected with *H. pylori,* although more detailed studies to confirm these findings are required.

In conclusion, we have demonstrated that *H. pylori* induces the expression and activation of PPARδ in a signaling axis that is dependent on p120 and β-catenin. These events require the *cag* secretion system effectors, CagA and peptidoglycan, which act through two independent pathways. PPARδ activation promotes cell proliferation in response to *H. pylori* infection of gastric epithelial cells in a three-dimensional matrix system, which may be mediated by the expression of Cyclin E1. Since PPARδ regulates a multitude of host responses such as cell turnover and inflammation, activation of this receptor may not only contribute to varying levels of cellular turnover within gastric tissue, but also to the diverse pathologic outcomes associated with *H. pylori* infection.

CHAPTER IV

HOST SIGNALING PATHWAYS THAT MEDIATE CELL MIGRATION IN RESPONSE TO *HELICOBACTER PYLORI*

Summary

Gastric adenocarcinoma is the second leading cause of cancer-related death worldwide, and chronic gastritis induced by *Helicobacter pylori* is the strongest known risk factor for this malignancy. One *H. pylori* virulence constituent that augments cancer risk is the *cag* secretion system, which functions to translocate bacterial effectors, such as CagA and peptidoglycan, into host cells. The interaction of these bacterial effectors with host cell proteins eventuates in activation of signal transduction pathways. PI3K signaling is activated in gastric cancer and by *H. pylori*, but the relationship between this cascade and *H. pylori*-induced cellular responses with carcinogenic potential remains unclear. We defined the molecular pathways mediating *H. pylori*-stimulated cell motility, a phenotype acquired by cells in epithelial to mesynchemal transition. *H. pylori* enhanced migration of gastric epithelial cells in a PI3K- dependent manner. These events were dependent upon structural components of the *H. pylori cag* secretion system, and peptidoglycan translocation. These results indicate that PI3K- signaling regulates pathophysiologic responses to *H. pylori* infection that may lower the threshold for gastric carcinogenesis.

Introduction

Helicobacter pylori is a Gram-negative bacterial pathogen that selectively colonizes the gastric epithelium of approximately half of the world's population. Chronic gastritis induced by this pathogen persists for decades and a biological consequence of long-term inflammation is an increased risk of developing gastric adenocarcinoma [219, 221]. Although *H. pylori*-induced gastritis is the strongest known risk factor for gastric cancer, only a fraction of colonized individuals ever develop neoplasia, and enhanced cancer risk is mediated by strain-specific bacterial factors and/or inflammatory responses governed by host genetic diversity. The *cag* pathogenicity island (*cag* PAI) is a well-characterized virulence locus that is present in approximately 60% of U.S. *H. pylori* strains [44]. Although all *H. pylori* strains induce gastritis, strains that harbor the *cag* PAI (cag^+) significantly augment the risk for severe gastritis, atrophic gastritis, and distal gastric cancer compared to those strains that lack the *cag* island (cag^-) [221].

Several *cag* genes, such as *cagE,* encode components of a type IV secretion system that exports bacterial proteins into host cells. The terminal product of the *cag* island, CagA, is translocated into gastric epithelial cells following bacterial attachment [20, 208, 292]. CagA subsequently undergoes tyrosine phosphorylation by Src and Abl kinases, and phospho-CagA alters gastric cell morphology and aberrantly activates signaling molecules such as SHP-2 [128, 270]. Unphosphorylated CagA can also exert effects within host cells such as altering cell polarity and activating β-catenin, responses that have been implicated in carcinogenesis [10, 24, 98].

Signal transduction pathways activated in response to bacterial contact play an important role in *H. pylori* pathogenesis. Phosphatidylinositol 3-kinase (PI3K) is an integral component of a signal transduction pathway that regulates host cellular responses altered in tumorigenesis. In addition to AKT, the primary target of PI3K, Rho-GTPases Rac1 and Cdc42 are influenced by PI3K signaling. These Rho family GTPases are critical mediators of cytoskeletal dynamics and cell-cell adhesion. Activation of Rac1 and Cdc42 results in actin polymerization and formation of lamellipodia and filopodia at the leading edge of the cell, events that are required for the migratory phenotype of cells. Of note, EGFR transactivation increases intestinal epithelial cell motility in a PI3K- and Src-dependent manner [105].

Cellular migration plays an important role in the invasive potential and metastatic growth of cancers. Although *H. pylori* can increase gastric epithelial cell migration, the mechanisms required for this response are not clearly defined [5, 192, 294]. Specifically, injection of CagA is required for the development of an elongated cell morphology known as the "Hummingbird" phenotype; however, cell migration requires a functional type four secretion system, but not CagA [4, 191, 332]. These observations suggest another translocated bacterial factor may stimulate specific signal transduction pathways leading to cell migration in response to *H. pylori.* Indeed, in addition to CagA, components of peptidoglycan are translocated into host cells by the *cag* secretion system where they are sensed by the intracellular pattern recognition receptor Nod1, which activates NF-κB and induces the production of pro-inflammatory cytokines such as IL-8. In chapter II, we have demonostrated *H. pylori* peptidoglycan is required for induction of

PI3K. Therefore, we investigated the role of PI3K signaling in cell migration in response to *H. pylori* and the bacterial factors required for these events. We demonstrate that *H. pylori* induces the PI3K axis, which promotes gastric cell migration. These processes were found to be dependent on the *cag* secretion system and peptidoglycan translocation.

Experimental procedures

Cell Culture and Reagents.

AGS human gastric epithelial cells were grown in RPMI medium 1640 (GIBCO/BRL) with 10% FBS (Sigma) and 20 μg/ml gentamicin (GIBCO/BRL) under 5% CO_2 air at 37°C. Pharmacological inhibitors LY294002 (Cell Signaling Technology), AG1478 (Calbiochem), PP2 (Calbiochem), AG1295 (Calbiochem), and STI-571 (LC Laboratories) were used at concentrations of 50 μM, 600 nM, 10 μM, 50μM and 10μM, respectively. For Western immunoblot analysis, AGS cells were plated at 5 x 10^6 cells/well in 10cm^2 plates in 10 mL culture medium. For cell migration assays, 5 x 10^5 cells were plated in 35 mm culture dishes in 2 mL medium.

H. pylori strains.

The *H. pylori cag*$^+$ rodent-adapted strain 7.13 was grown in *Brucella* broth with 5% FBS for 18 hours, harvested by centrifugation, and were added to gastric cells at a bacteria-to-cell ratio of 100:1. Isogenic *cagA*$^-$, *cagE*$^-$, and *slt*$^-$ null mutants were constructed within strain 7.13 by insertional mutagenesis using *aphA* and were selected with kanamycin (25 μg/ml) as described previously [222].

Western Blot Analysis.

Gastric cell lysates were harvested and fractionated using Qproteome Cell Compartment Kit (Qiagen). Proteins (30 μg) were separated by 10% SDS-PAGE and transferred to poly-vinylidene difluoride membranes (Pall). Membranes were blocked in 5% nonfat dry

milk in Tris-buffered saline with 0.05% Tween 20 (TBST), incubated for 24 hours with a purified rabbit polyclonal anti-Rac1 antibody (1:1000 dilution; Santa Cruz Biotech) and a monoclonal mouse anti-GAPDH antibody (1:2000 dilution; Santa Cruz Biotech). Goat anti-rabbit (1:5000 dilution; Santa Cruz Biotech) or goat anti-mouse (1:5000 dilution; Santa Cruz Biotech) horseradish peroxidase-conjugated secondary antibodies were used followed by enhanced chemiluminescence detection following the manufacturer's instructions (Perkin Elmer). Immunoblots were quantified with the GeneTools Software (Syngene).

Cell Migration Analysis.

Confluent AGS cell monolayers in plates coated with 2.5 μg human fibronectin (BD Bioscience) were pre-incubated with pharmacological inhibitors for one hour. Eight circular wounds were generated in each plate using a rotating silicon tip [62]. *H. pylori* was then added to the cells and wound images were taken at zero, six and sixteen hours post infection using Q-Capture Imaging Software. Areas were measured using Image J software (NIH).

Immunofluorescence

Gastric epithelial cells were cultured on glass cover slides, and cells treated with or without *H. pylori* in conjunction with the PI3K inhibitor were washed twice with PBS, permeabilized, and fixed with ice-cold methanol at –20 °C as described previously. Slides were incubated in 3% BSA (Sigma) for 10 min and then incubated with rabbit anti-

β-catenin antibody (1:100; Sigma) overnight at 4 °C. Washed slides were then incubated with goat anti-rabbit AlexaFluor 488 (1:200; Invitrogen) at room temperature for 30 min.

Statistical Analysis.

All experiments were performed on at least three independent occasions. Statistical analysis was performed by Student's t test and ANOVA using Prism Graph Pad. A P-value < 0.05 was defined as statistically significant.

Results

PI3K signaling is required for cell migration in response to H. pylori.

Colonic epithelial migration is dependent on PI3K and Src activation [81]. We have recently demonstrated that the carcinogenic *H. pylori* strain 7.13 induces PI3K activation in gastric epithelial cells (**Figure 10**). To determine if *H. pylori* promotes cell migration in a PI3K-dependent manner, AGS cells were treated with the PI3K inhibitor LY294002 and infected with *H. pylori.* Wounds were then induced and measured over time using time-lapse microscopy. Inhibition of PI3K did not significantly alter cell motility in uninfected cells (**Figure 21A, 21B**). *H. pylori* significantly increased wound healing compared to uninfected cells, but this was abolished by inhibition of PI3K (**Figure 21A, 21B**). Treatment of cells with EGFR (AG1478) and Src (PP2) inhibitors also blocked migration in response to *H. pylori* (**Figure 21C**), which mirrored results investigating the effects of *H. pylori* on PI3K-AKT activation (**Figure 13**). Since PP2 can also inhibit PDGFR signaling, we repeated migration assays in the presence or absence of the specific PDGFR inhibitor AG1295. *H. pylori*-induced cell migration was unchanged in the presence of the PDGFR inhibitor (**Figure 21C**), indicating that Src likely plays a role in cell motility that is induced by *H. pylori*.

Cell migration in response to H. pylori also requires Rac1.

H. pylori and PI3K can also activate the small GTPase Rac [54, 234], an important regulator of the migratory phenotype of cancer cells. Therefore, we next investigated whether Rac activation influenced *H. pylori*-induced wound closure by repeating wound-healing assays in the presence of a specific Rac1 inhibitor (NSC23766).

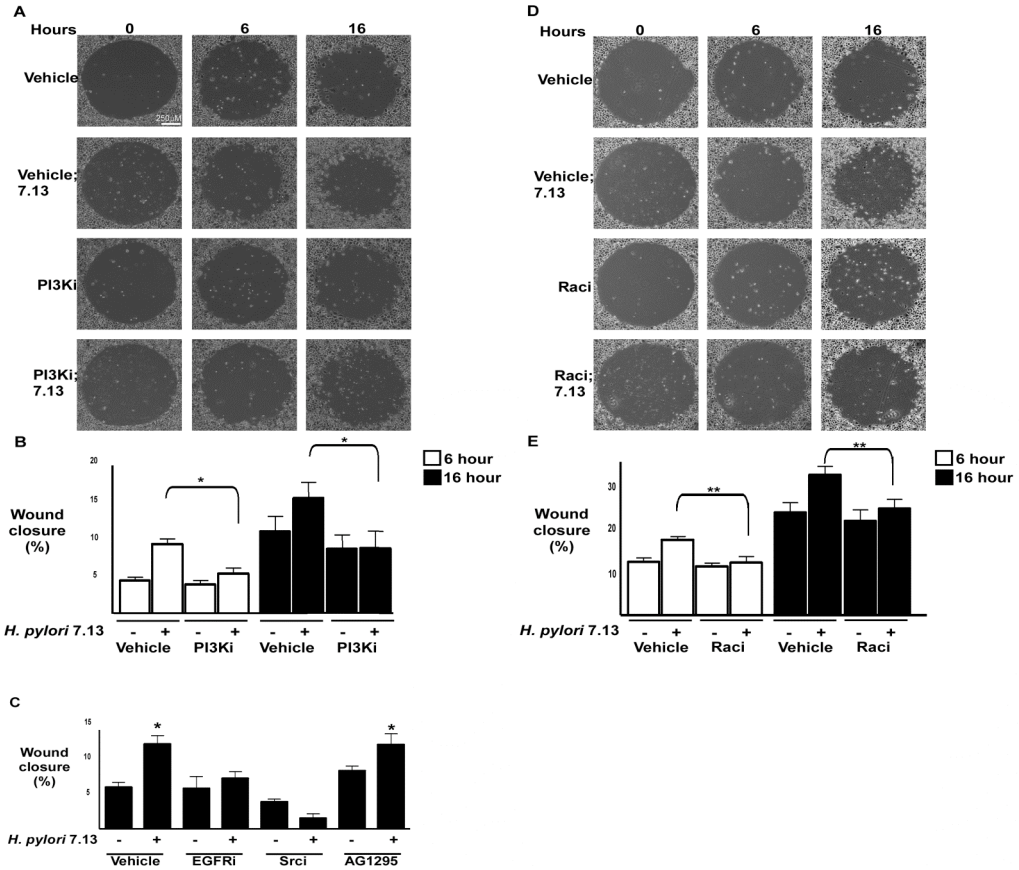

Figure 21. Activation of PI3K-AKT and Rac mediates *H. pylori*-induced cell migration. ***(A)*** AGS cells were grown to confluency and incubated with the PI3K inhibitor LY294002 (50 μM) or vehicle alone (DMSO) for one hour. A wound was then introduced into the cell monolayer and medium or *H. pylori* strain 7.13 was added. Wound areas were measured at zero, six and sixteen hours post-infection. ***(B)*** Quantification of wound closure for each treatment group in experiments performed on at least 5 independent occasions. (-), cells incubated without *H. pylori*. Error bars = SEM. * $P < 0.04$ vs AGS cells infected with *H. pylori* strain 7.13 in the presence of the PI3K inhibitor LY294002 at both six and 16 hours. ***(C)*** AGS cells were grown to confluency and incubated with 600 nmol/L AG1478, 10 μmol/L PP2, or 50 μmol/L AG1295, or vehicle alone (DMSO) for one hour. A wound was then introduced into the cell monolayer and medium or *H. pylori* strain 7.13 was added. Wound areas were measured at zero and six hours post-infection. Quantification of wound closure for each treatment group in experiments performed on at least 3 independent occasions is shown. (-), cells incubated without *H. pylori*. Error bars = SEM. * $P < 0.005$ vs uninfected AGS cells alone or in the presence of AG1295. ***(D)*** AGS cells were grown to confluency and incubated with the Rac1 inhibitor NSC23766 (50 μM) or vehicle alone (water) for one hour. A wound was then introduced into the cell monolayer and medium or *H. pylori* strain 7.13 was added. Wound areas were measured at zero, six and sixteen hours post-infection. ***(E)*** Quantification of wound closure for each treatment group in experiments performed on at least 5 independent occasions. (-), cells incubated without *H. pylori*. Error bars = SEM. ** $P < 0.01$ vs AGS cells infected with *H. pylori* strain 7.13 in the presence of the Rac inhibitor NSC23766 at both six and 16 hours.

Inhibition of Rac did not significantly alter cell motility in uninfected cells (**Figure 21D, 21E**). Similar to results seen with PI3K inhibition, *H. pylori*-induced cell migration was completely abolished in the presence of Rac inhibition (**Figure 21D, 21E**). These results indicate that *H. pylori* promotes gastric epithelial cell migration via a PI3K, Src and Rac-dependent pathway, likely transduced by upstream signaling from EGFR transactivation.

Rac membrane localization is reduced by PI3K inhibition.

Localization of Rac to the cell membrane is indicative of GTP-loaded, active Rac. To more firmly implicate the requirement of PI3K in Rac activation, we performed cell fractionation of AGS gastric epithelial cells co-cultured with wild-type *H. pylori* strain 7.13 in the absence or presence of PI3K inhibition. Western blot analysis revealed an increase in Rac membrane localization in response to infection, but this was abolished in the presence of PI3K inhibition (**Figure 22**). Similar results were seen with immunofluorescence of Rac1 in *H. pylori*-infected cells (data not shown). These results support the hypothesis that *H. pylori*-induced PI3K leads to Rac activation and the subsequent motogenic response.

H. pylori-induced cell migration is dependent on cagE and peptidoglycan.

Having demonstrated in Chapter II that *cagE* and peptidoglycan are required for *H. pylori*-induced PI3K-AKT activation (**Figure 11**), we next determined the role of these bacterial factors in cell migration. Similar to the patterns observed for AKT activation (**Figure 11**), cell migration was significantly decreased following infection with the *cagE*$^-$ or *slt*$^-$ mutants compared to wild-type 7.13 (**Figure 23**).

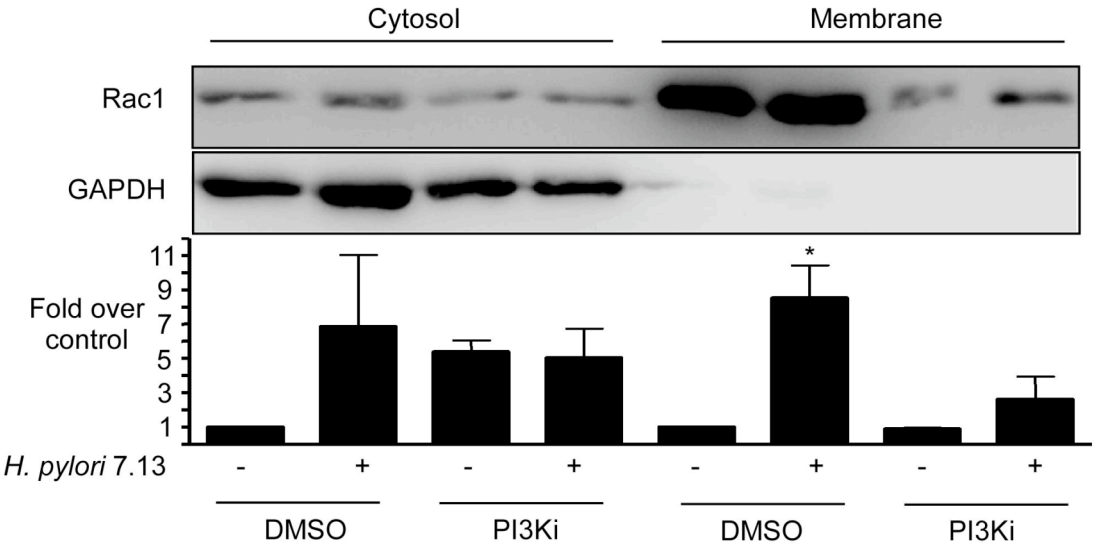

Figure 22. *H. pylori* alters subcellular localization of Rac via PI3K. AGS cells were cocultured with *H. pylori* strain 7.13 (MOI = 100) or medium alone for 16 hours. Total protein was extracted and subjected to subcellular fractionation, and analyzed by Western blot using an anti-Rac antibody. Representative blots are shown. Anti-GAPDH antibodies served as normalization controls for purification of cytosolic and membrane fractions. Densitometric analysis of multiple Western blot repetitions; graph represents fold Rac1 expression in infected versus uninfected cells. Error bars, SEM. *$p < 0.05$.

These results indicate that PI3K signaling and *cag*-mediated peptidoglycan translocation mediate *H. pylori*-induced cell migration.

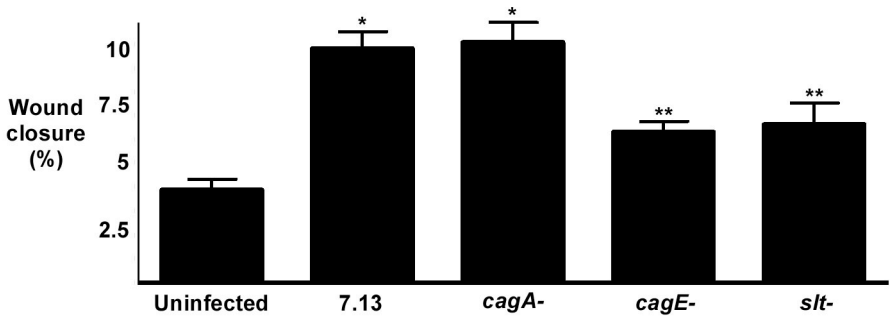

Figure 23. *H. pylori*-induced cell migration is dependent on the *cag* pathogenicity island and peptidoglycan. AGS cells were grown to confluency and a wound was introduced into the monolayer. Medium, *H. pylori* strain 7.13, or isogenic *cagA*$^-$, *cagE*$^-$, or *slt*$^-$ null mutant derivatives were then added at bacteria/cell ratios of 100:1. Wound areas were measured at time zero and six hours post-infection. Quantification of wound closure is shown for each treatment group in experiments performed on at least 3 occasions. Error bars = SEM. * $P < 0.007$ vs uninfected control; ** $P < 0.007$ vs AGS cells infected with *H. pylori* strain 7.13.

Discussion

Investigations into mechanisms through which *H. pylori* promotes gastric cancer have demonstrated that disease risk involves specific interactions between pathogen and host, which are dependent on strain-specific bacterial constituents and induced host effectors. *H. pylori cag*$^{+}$ strains are isolated significantly more frequently from persons with atrophic gastritis and distal gastric cancer than from subjects with gastritis alone [36, 68, 157] and genes within the *cag* island are necessary for induction of epithelial cell responses related to pathogenesis [145, 182, 202, 313].

Increased migration is a trait acquired by cells in the transition to a metastatic phenotype. For metastasis to occur, malignant cells must detach from neighboring cells and migrate into adjacent tissue, a process similar to epithelial-mesenchymal transition (EMT), which allows stationary epithelial cells to become motile. Disruption of intercellular junctions (e.g. adherens junctions) is required for EMT to occur and *H. pylori* can disrupt junctional complexes. Ectopic expression of CagA in epithelial cells results in a loss of polarity and cell-cell adhesion, as well as extension of migratory pseudopodia and acquisition of an invasive phenotype [24]. However, CagA-independent disruption of the adherens junction protein E-cadherin has also been reported [333]. PI3K may play a role in this process as this molecule has been implicated in the migratory and metastatic phenotype of carcinoma cells. PI3K can interact with and be activated by E-cadherin [220], and PI3K activation up-regulates Snail, a transcriptional repressor of E-cadherin, through phosphorylation of GSK3β [18]. PI3K can also activate the small GTPase Rac

[155, 233] and integrin-linked kinase [76], molecules which are important regulators of the migratory and invasive phenotype of cancer cells. Integrin signaling can induce EMT via activation of Focal Adhesion Kinase (FAK), which occurs through a PI3K-Rac signaling cascade [276]. Recently, FAK activation by *H. pylori* has been shown to result in the production of stress fibers; however, in this system, inhibition of PI3K had no effect on these changes in cell phenotype [299].

In macrophages, *H. pylori cag*$^{+}$ strains activate PI3K, leading to actin polymerization and delayed phagocytosis [8]. Our current studies, which focused on epithelial cells, demonstrate a dramatic reduction in *H. pylori*-induced cell migration in the presence of PI3K inhibitors, suggesting that PI3K may also mediate actin dynamics in gastric epithelial cells, likely through Rac1 activation. We determined that cell migration was not affected by loss of CagA, but did require a functional type IV *cag* secretion system and peptidoglycan translocation. Our results are concordant with other studies demonstrating that certain signal transduction pathways, such as those leading to NF-κB, JNK, and activator protein-1 (AP-1) activation, are *cag* secretion system-dependent, but CagA-independent [53, 113, 202]. Our results differ from studies demonstrating that CagA is required for a full motogenic response to *H. pylori* through its interactions with the c-Met receptor and subsequent MEK/ERK signaling [52]. We speculate that these differences may be due in part to the use of different strains as well as different techniques to assess cell migration However, Al-Ghoul *et al.* have shown that *H. pylori* mutants that do not translocate CagA can still stimulate cell motility, suggesting that additional factors translocated by the type IV secretion system affect *H. pylori*-dependent

motility [5]. Consistent with these findings, our results indicate that the peptidoglycan translocation is required to promote cell migration.

In summary, *H. pylori*-induced PI3K activation mediates cell migration of gastric epithelial cells, a phenotype related to carcinogenesis. These events require the *cag* secretion system and peptidoglycan as well as EGFR transactivation and Src activation in host cells. Taken together, these data present insights into the carcinogenic mechanisms underlying *H. pylori* infection.

CHAPTER V

CONCLUSIONS AND FINAL REMARKS

Summary

The discovery of *H. pylori* and the ultimate acceptance of work performed by Warren and Marshall in the 1980's have led to robust scientific pursuit in the fields of microbiology and cancer biology. Examination of mechanisms by which *H. pylori* interacts with gastric epithelium has provided a deeper understanding of ulcer disease and gastric adenocarcinoma, which remains the second leading cause of cancer- related deaths worldwide. New lines of *H. pylori* treatment have been developed because of these studies and eradication efforts have already begun to reduce rates of gastric cancer in developing countries.

Disease outcomes associated with *H. pylori* result from chronic inflammation induced by infection. Thus, the study of *H. pylori* has also provided a model for other organisms and diseases associated with chronic inflammation. Bacterial factors, survival mechanisms, and immune evasion make *H. pylori* uniquely adapted for life in the gastric niche. Reactive oxygen species produced during the host response to *H. pylori,* in conjunction with deregulated signaling pathways within epithelial cells, may lead to the degeneration of normal cell function and initiation/promotion of gastric carcinogenesis. Though inflammation is induced by all *H. pylori* infections, the vast majority do not result in

cancer, underscoring the importance of defining the strain-specific and host factors that result in tumorigenesis.

Insight into signaling cascades that are deregulated within gastric epithelial cells by *H. pylori* is critical to understanding the pathogenesis of this organism. Translocation of CagA by the *cag* secretion system induces activation of c-Met, ERK1/2 and β-catenin [10, 19, 99]. *H. pylori* peptidoglycan activates NOD1-dependent NF-κB and unpublished data suggest p38 activation also requires peptidoglycan components [319]. Alteration of these signaling constituents in response to *H. pylori* may facilitate oncogenic changes. For instance, activation of survival pathways by *H. pylori* may result in self-sufficiency in growth signals and insensitivity to anti-growth signals. In concordance, *H. pylori cag*$^{+}$ strains selectively enhance proliferation and attenuate apoptosis in human mucosa and rodent models of infection [226, 254]. The goal of this dissertation was to investigate new mechanisms of pathogenesis and signaling pathways regulated by *H. pylori* that mediate its carcinogenic effects. Specifically, how does *H. pylori* alter signal cascades that contribute to increasing cell survival and decreasing rates of apoptosis? These two events favor tumorigenesis and are important in lowering the threshold for carcinogenesis.

In Chapter II, activation of the PI3K signaling cascade was identified to be a critical regulator of resistance to apoptosis in *H. pylori*-infected gastric epithelial cells. At the time this project was initiated, there was only one report describing activation of PI3K in *H. pylori*-infected macrophages [7]. We first demonstrated that carcinogenic strains of

H. pylori activate the PI3K-AKT signaling axis in gastric epithelial cells (**Figure 10**), and requires upstream signaling from EGFR and Src kinase (**Figure 13**), two signaling components known to be activated by *H. pylori* [146, 291]. Furthermore, our *in vitro* results indicated that induction of PI3K-AKT signaling by *H. pylori* requires translocation of peptidoglycan via the *cag* secretion apparatus (**Figure 12**). These results reveal a previously unrecognized consequence of translocation of this *cag* island substrate, as the only defined role to date of *cag*-mediated peptidoglycan delivery is NOD1-dependent induction of IL-8 secretion by gastric epithelial cells [319]. When we examined regulation of apoptosis by *H. pylori,* we found that activation of PI3K and downstream AKT results in reduced rates of apoptosis and apoptotic resistance of gastric cells (**Figure 14**). Therefore, activation of the PI3K-AKT signaling axis may contribute to cell changes that provide insensitivity to anti-growth signals and allow progression to a cancerous state.

When performing Propidium iodide staining and flow cytometric analysis of the cell cycle in gastric cells co-cultured with *H. pylori,* we also found that infection induced two distinct cell populations (data not shown). *H. pylori* did induce an appreciable amount of cell death; however, there was also an increase in the number of cells in the S phase of the cell cycle, indicative of replicating cells. We hypothesized that the anti-apoptotic response mediated by *H. pylori*-induced PI3K activation may allow for a subset of gastric cells to survive and proliferate. Therefore, in Chapter III, we elucidated additional signaling pathways that are activated in response to *H. pylori* infection that mediate cell proliferation, a phenotype acquired by cancerous cells to propagate. We investigated the

role of PPARδ, a ligand activated transcription factor that affects a multitude of normal cell functions. Work in colorectal cancer has highlighted the ability of PPARδ to promote carcinogenesis when aberrantly activated [120, 324], though it is clear that more studies are needed to delineate the specific mechanisms underpinning these observations.

We first demonstrated that *H. pylori* induces the expression and functional activation of PPARδ in gastric epithelial cells (**Figure 15**). Previously, our lab had shown that the adherens junction component p120, is required for relief of transcriptional repression of the β-catenin target gene, *mmp7,* which is known to play a critical role in tumorigenesis [210]. We have now identified PPARδ as another specific target of the p120/ β-catenin pathway (**Figure 16**). Furthermore, we demonstrated that loss of both CagA and Slt completely abolishes β-catenin and subsequent PPARδ activation (**Figure 17**). Previous reports have shown CagA to be solely responsible for β-catenin activation; however, these studies used AGS gastric epithelial cells that contain constitutively activated β-catenin and lack E-cadherin expression [99, 297], or cells inducibly expressing CagA [197]. These artificial systems may not account for additional factors that are present only in live bacterial infection. Our current findings using live infection of MKN28 cells that express β-catenin at the cell membrane and form functional adherens junctions, indicate that multiple *H. pylori* constituents can mediate β-catenin activation (**Figure 17**). We next investigated whether PPARδ mediates a proliferative response in host cells using dominant negative and RNA silencing techniques. With this, we found that *H. pylori* promotes epithelial cell hyperproliferation in a PPARδ-dependent manner (**Figure 18**). Importantly, these results mimic the proliferative response to *H. pylori* seen *in vivo*

[226, 254]. We also found that *H. pylori* induces the expression of a critical cell cycle mediator, *cyclin E,* in a PPARδ-dependent manner and these *in vitro* findings occur in an *ex vivo* culture system that recapitulates the cellular organization of the stomach (**Figures 19 and 20**). Increased proliferation without a concordant increase in apoptosis may therefore contribute to the heightened retention of mutagenized cells, which over decades may increase the risk for gastric cancer.

Though cellular migration is continuously occurring in normal tissues during development, the process is highly controlled. Cancerous cells require the ability to be motile for migration and invasion to distant sites, similar to epithelial-mesenchymal transition (EMT), which allows stationary epithelial cells to become motile [1]. In Chapter IV, we report that *H. pylori* induces epithelial cell migration that is dependent on Rac activation downstream of PI3K (**Figures 21 and 22**). Similar to patterns observed in PI3K-AKT activation in response to isogenic mutants of *H. pylori* (**Figures 11 and 12***),* we found that cell migration requires peptidoglycan translocation via the *cag* secretion system (**Figure 23**). It is possible the subset of cells that evade apoptosis and proliferate in response to *H. pylori* undergo a motile response. This, in conjunction with breakdown of cell-cell contacts, may lead to the release of nutrients from paracellular spaces to enhance survival of *H. pylori*, and maintenance of an invasive host cell phenotype, which over time may result in metastatic gastric carcinoma cells.

Taken together, the studies in this dissertation provide a mechanism through which *H. pylori* alters rates of epithelial turnover in the gastric niche through activation of

signaling pathways within host cells that potentially drive tumorigenesis (**Figure 24**). An anti-apoptotic response mediated by PI3K activation, in the presence of increased proliferation mediated by PPARδ, increases the risk of retaining mutagenized gastric epithelial cells in the presence of *H. pylori* induced gastritis. These studies have therefore defined effectors that directly mediate host responses related to carcinogenesis, providing a foundation for future work of understanding the full circuitry of these pathways and the biological consequences of perturbing them.

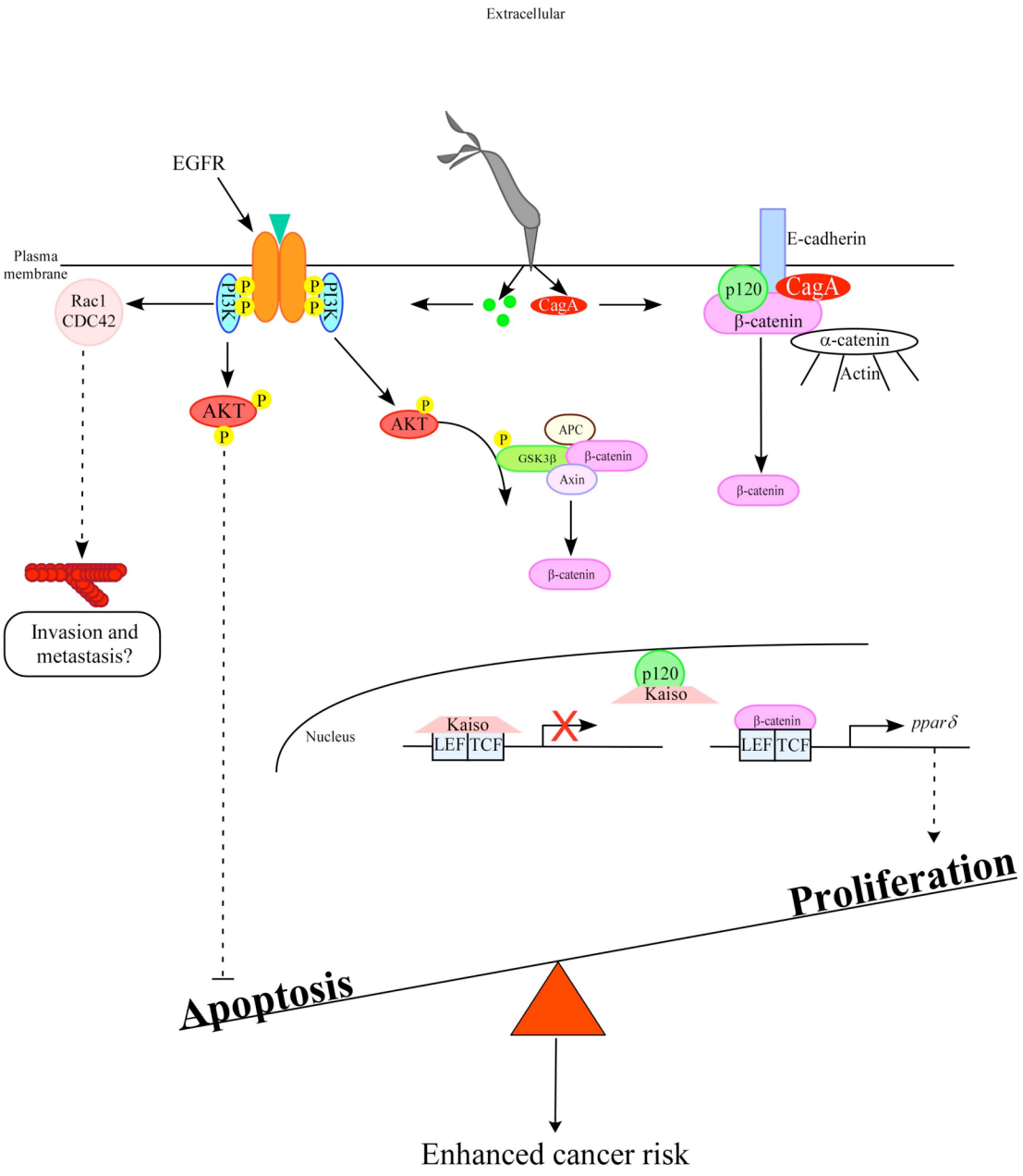

Figure 24. Model of dysregulated cellular turnover in response to *H. pylori*. Translocation of *H. pylori* peptidoglycan mediates PI3K activation, which is required for decreasing rates of apoptosis and promoting cell migration. Translocation of CagA and peptidoglycan promote β-catenin accumulation in the cytosol and nucleus, which acts to drive PPARδ upregulation in a p120-dependent manner. PPARδ activation results in enhanced cell proliferation, which in the presence of decreased apoptosis, promotes accumulation of gastric cells damaged by *H. pylori*-induced gastritis.

Preliminary Data and Future Directions

Activation of PI3K-AKT occurs in biopsies obtained from H. pylori-infected individuals. Our *in vitro* data demonstrate that activation of the PI3K-AKT signaling pathway within *H. pylori*-infected gastric epithelial cells is important in regulation of cell survival and apoptosis. It will be important to take these studies into *in vivo* models of infection. We have now performed immunohistochemistry for Stathmin, a marker of activated PI3K signaling [259], on biopsies from *H. pylori*-infected humans. Our preliminary data suggest there is an increase in the number of Stathmin-positive cells in humans infected with *H. pylori* (**Figure 25**), reflecting PI3K activation, which likely contributes to the pathogenesis of *H. pylori in vivo.* Recent published reports now support these findings by demonstrating increased AKT phosphorylation, indicative of PI3K activation, in *H. pylori*-infected human biopsies [301]. It will be important to test the hypothesis that PI3K-AKT contributes directly to *H. pylori* pathogenesis *in vivo* using AKT deficient mice. These mice are difficult to breed in order to obtain adequate numbers for statistical power due to high rates of embryonic death and poor survival post-birth [241]. In addition, there are three isoforms of AKT and it is currently not known which of these *H. pylori* activates specifically. It will therefore be necessary to further delineate the exact isoforms of AKT that are activated by *H. pylori* and choose the corresponding knockout mice for infection. We hypothesize that AKT knockout mice will develop significantly less serious sequelae of *H. pylori* infection compared to wild-type mice. Because it may not be feasible to breed adequate numbers of mice for infection, an alternative strategy is

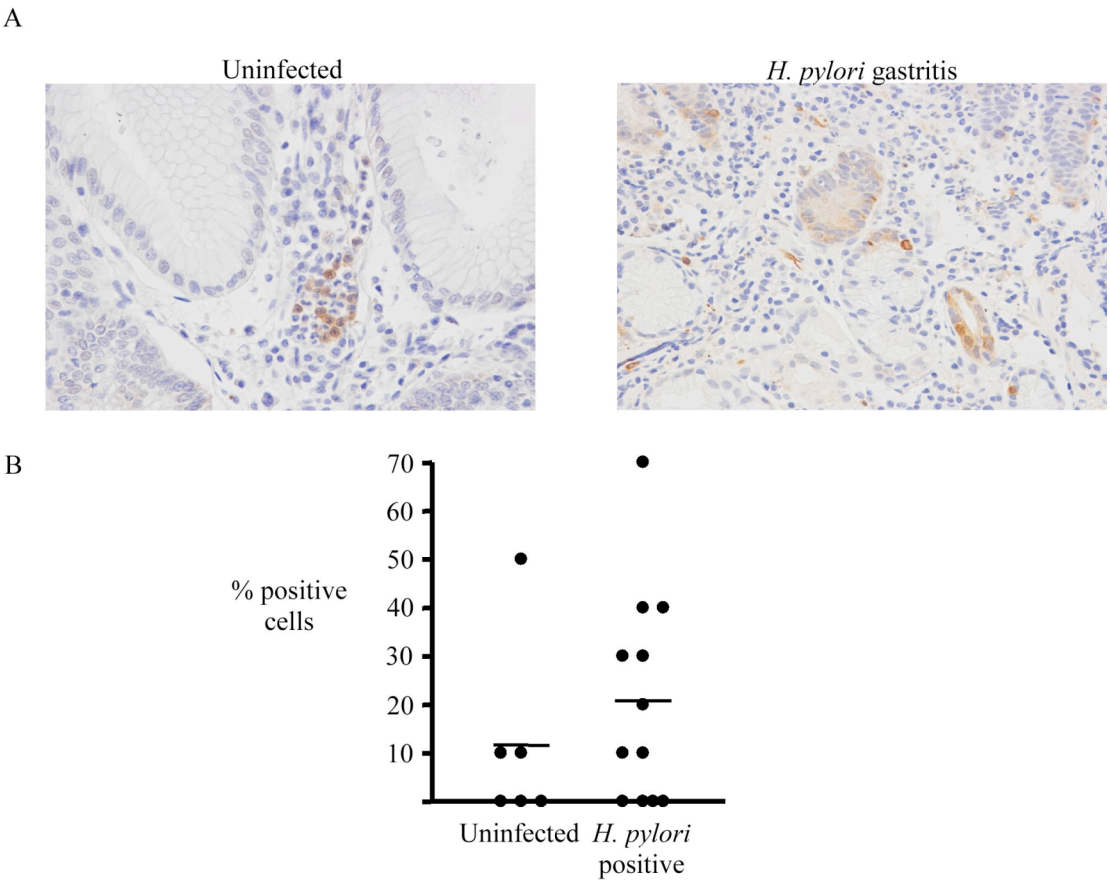

Figure 25. PI3K activation is increased in *H. pylori*-positive biopsies. (*A*) Representative staining for Stathmin is shown for uninfected and *H. pylori*-infected persons. ***(B)*** Gastric epithelial cells with Stathmin staining as assessed by immunohistochemistry were quantified by an observer unaware of *H. pylori* status. Results are expressed as the percentage of cells per sample (10 high-powered fields/sample examined) with detectable Stathmin staining.

to obtain primary gastric glands to examine carcinogenic cell responses to *H. pylori* in an *ex vivo* system. These experiments would require long-term efforts, but would be useful to further support the role of PI3K, and specifically downstream AKT activation, in *H. pylori*-mediated carcinogenesis.

Determine expression of PPARδ in H. pylori-infected humans and gerbils.

Our *in vitro* and *ex vivo* studies described in Chapter III indicate that *H. pylori* activates PPARδ. Because there are no studies to date examining PPARδ expression in *H. pylori*-infected humans or rodents, it will be necessary to next determine if our findings are relevant in a rodent model of gastric cancer. We have began to perform these experiments by quantifying levels of PPARδ expression in Mongolian gerbils infected with wild-type *H. pylori* strain 7.13 or the isogenic *cagA*$^-$, *cagE*$^-$, *slt*$^-$, or *cagA*$^-$/*slt*$^-$ mutants. We have now obtained inflammation and injury scores from gerbils infected for 6 and 12 weeks. As previously demonstrated [100], all gerbils infected with *H. pylori* strain 7.13 developed significantly higher inflammatory scores compared with uninfected controls (**Figure 26**). This response was significantly attenuated in gerbils infected with *cagA*$^-$, *cagE*$^-$, *slt*$^-$, or *cagA*$^-$/*slt*$^-$ isogenic mutants compared with those infected with parental wild-type 7.13. Additionally, gastric adenocarcinoma was present only in 7.13-infected gerbils by 6 weeks post-infection. We will next perform immunohistochemistry on paraffin-embedded sections to detect and quantify levels of PPARδ and Cyclin E1. Though IHC has not been optimized to date, we expect to see increased levels of PPARδ and Cyclin E1 in gerbils infected with wild-type *H. pylori* strain 7.13 and lower levels in gerbils infected with mutant strains. Additionally, we will examine levels of PPARδ in

H. pylori-positive and negative human biopsies. These data will be necessary to support our *in vitro* findings.

Since the PPARδ agonist GW501516 has previously been shown to promote carcinogenesis in a mouse model of colon cancer [120], it will also be interesting to determine if administration of this agonist can accelerate *H. pylori*-initiated gastric cancer. Gerbils can be infected with *H. pylori* and co-administered 0.5% carboxymethylcellulose containing either GW501516 or vehicle by gavage feeding. A potential result is that GW501516 will accelerate injury and proliferation in gerbils as we have demonstrated *in vitro*. These findings would be exciting as they would support our hypothesis that PPARδ activation mediates *H. pylori*-induced carcinogenesis.

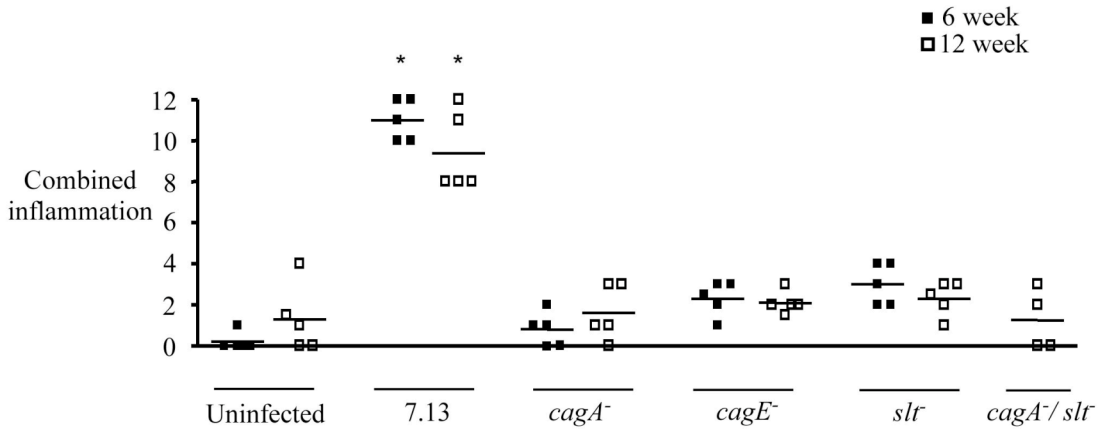

Figure 26. Severity of inflammation and injury within the gastric mucosa of Mongolian gerbils inoculated with *H. pylori* is dependent on the *cag* island effector molecules. Combined antral and corpus inflammation in gerbils infected with broth alone or with *H. pylori* strains 7.13, 7.13 *cagA*$^-$, 7.13 *cagE*$^-$, 7.13 *slt*$^-$, or 7.13 *cagA-/slt*$^-$ for 6 or 12 weeks. Inflammation was determined by histologic examination, and data are presented as scatter plots with mean values. *$p < 0.05$ versus gerbils inoculated with broth alone.

PPARδ likely contributes to pathogenesis of H. pylori in vivo

In Chapter III, we demonstrated *H. pylori* induces PPARδ activation *in vitro* and *ex vivo,* requiring p120 in host cells and the bacterial effectors, CagA and peptidoglycan. PPARδ activation led to increased cell proliferation in response to infection, which may contribute to altering rates of epithelial turnover. In the future it will be necessary to move these studies into tractable *in vivo* systems of infection using PPARδ knockout mice. We propose to infect PPARδ$^{-/-}$ and wild-type C57Bl/6 mice with strain 7.13 and examine acute (6, 12, 24 hour) and chronic (1, 6, 12, 24 week) time points post challenge based on data indicating that 1) *H. pylori* alters epithelial signaling pathways acutely and 2) development of cellular responses with carcinogenic potential likely occurs over longer periods of infection. Because PPARδ regulates a multitude of host responses and carcinogenesis in some systems, it will be necessary to obtain inflammation and injury scores as well as rates of apoptosis (using cleaved- caspase 3) and proliferation (using Ki67). A potential result is that *H. pylori* infection of wild-type mice will initiate p120 mislocalization, and increased production of PPARδ and Cyclin E1, as we have seen in cell culture, which would support our hypothesis that PPARδ contributes to *H. pylori* pathogenesis. We anticipate that PPARδ$^{-/-}$ mice will exhibit decreased proliferation with acute and chronic *H. pylori* infection. We have performed a pilot experiment infecting wild-type and PPARδ$^{-/-}$ mice. The initial results indicate a trend towards less inflammation in infected PPARδ$^{-/-}$ mice compared with wild-type mice (**Figure 27**); however, a full study as proposed above is needed to determine the exact role of PPARδ *in vivo*.

Since levels of gastric damage differ among genetically distinct strains of mice infected with *H. pylori* [318], indices of *H. pylori*-induced, PPARδ-regulated gastric injury, may vary depending on the particular strain of mice examined. Though *H. pylori*-infected mice on a C57Bl/6 background are extremely useful for examining inflammatory responses, they rarely develop gastric cancer prior to 15 months post-challenge [253]. However, infection of hypergastrinemic INS-GAS mice with strain 7.13 eventuates in premalignant lesions and gastric cancer by 24 weeks post-infection [94]. Thus it will be important to generate PPARδ$^{-/-}$ mice on an INS-GAS background to define the role of PPARδ on the development of cancer. Infection of these mice will allow for the examination of inflammation, apoptosis and proliferation, and gastric carcinogenesis in response to *H. pylori* infection over a shorter time period. Taken together, these proposed experiments are essential to confirm that interactions between specific *H. pylori* constituents and PPARδ play an important role in host injury responses within the gastric niche.

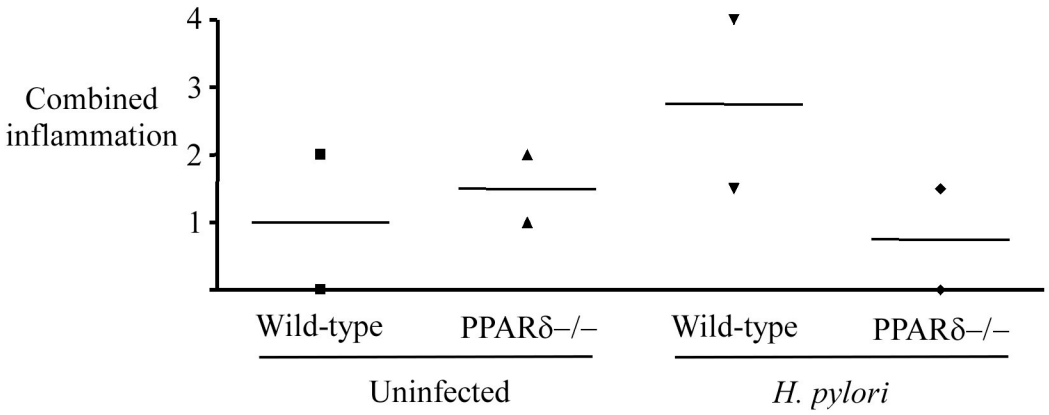

Figure 27. Infection of PPARδ$^{-/-}$ mice attenuates *H. pylori*-induced inflammation. Comparison of combined gastric antral and corpus inflammation in wild-type and PPARδ$^{-/-}$ mice infected with broth alone or *H. pylori* strain 7.13 for 8 weeks. *H. pylori*-induced inflammation appears to be attenuated in PPARδ$^{-/-}$ mice. Inflammation was determined by histological examination.

Final Remarks

The discovery of *H. pylori* and its role in gastric disease has resulted in exceptional advances in the fields of gastroenterology, microbiology, and cancer biology. The dynamic interactions that occur in *H. pylori*-induced carcinogenesis allow for the delineation of both microbial constituents and host factors that influence disease outcome and provide insight into how other chronic infections can lead to carcinogenesis.

The work presented in this dissertation adds to the understanding of these unique microbe-host interactions. In Chapter II, we describe the ability of *H. pylori* to activate PI3K-AKT signaling, which leads to apoptotic resistance of gastric epithelial cells. In Chapter III, we demonstrate that PPARδ, a downstream target of β-catenin and p120, is induced in response to *H. pylori* infection and plays a role in promoting cell proliferation. Collectively, activation of these host signaling components alters rates of cellular turnover. Decreased apoptosis in the presence of increased cell proliferation may allow for accumulation of mutations and enhanced cancer risk in the presence of *H. pylori*-induced gastritis. Additionally, we show in Chapter IV that activation of PI3K in response to infection results in enhanced cell migration, which may play a role in later stages of gastric cancer induced by *H. pylori*, such as in the metastatic spread of tumor cells. The host signaling components described are known to be oncogenic in other cancers, and we have now shown the potential role of PI3K and PPARδ in gastric cancer related to *H. pylori.*

Throughout the literature, it is clear that strain-specific virulence determinants play a major role in the pathogenic outcomes of *H. pylori*. The *cag* secretion system delivers CagA and peptidoglycan into host cells. CagA affects many signaling pathways and alters cell-cell contacts [10, 22]. Peptidoglycan translocation by the *cag* secretion system leads to NOD1-dependent proinflammatory responses in gastric epithelial cells [319]. In our current studies we have defined additional roles for the *cag* secretion substrates peptidoglycan and CagA, adding PI3K and PPARδ to the list of host signaling pathways selectively altered by *cag*$^+$ strains of *H. pylori* to induce epithelial responses that play a role in cancer promotion.

Activation of PI3K and PPARδ contributes to survival, motility and proliferation, which are all essential aspects of tumorigenesis. Induction of these signaling constituents by *H. pylori* may act as a promoting agent, stimulating precancerous-cell survival and proliferation. As infection persists for decades, reactive oxygen species and other DNA-damaging agents produced in the chronic inflammatory response to *H. pylori* may subsequently lead to genetic mutations and instabilities as precancerous cells divide. Prolonged stimulation of these signaling pathways may allow gastric cells to become capable of autonomous cell growth, and progress to gastric adenocarcinoma. Additional experiments are required to fully understand how *H. pylori* alters the gastric epithelium to promote carcinogenesis; however, by defining signaling components that are altered in cells infected with *H. pylori,* we may be able to define therapeutic targets for gastric cancer, and examination of bacterial factors that enhance pathogenicity may allow physicians to more accurately target infected individuals with enhanced cancer risk. Due to the extensive morbidity and mortality related to *H. pylori,* there have been

considerable efforts focused on defining mechanisms by which this pathogen induces disease and the work presented in this dissertation provides insight into the dynamic interplay between *H. pylori* and host, which may lead to meaningful clinical applications in the future.

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