Review

Structure and function of *Helicobacter pylori* CagA, the first-identified bacterial protein involved in human cancer

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Abstract: Chronic infection with *Helicobacter pylori cagA*-positive strains is the strongest risk factor of gastric cancer. The *cagA* gene-encoded CagA protein is delivered into gastric epithelial cells via bacterial type IV secretion, where it undergoes tyrosine phosphorylation at the Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs. Delivered CagA then acts as a non-physiological scaffold/hub protein by interacting with multiple host signaling molecules, most notably the pro-oncogenic phosphatase SHP2 and the polarity-regulating kinase PAR1/MARK, in both tyrosine phosphorylation-dependent and -independent manners. CagA-mediated manipulation of intracellular signaling promotes neoplastic transformation of gastric epithelial cells. Transgenic expression of CagA in experimental animals has confirmed the oncogenic potential of the bacterial protein. Structural polymorphism of CagA influences its scaffold function, which may underlie the geographic difference in the incidence of gastric cancer. Since CagA is no longer required for the maintenance of established gastric cancer cells, studying the role of CagA during neoplastic transformation will provide an excellent opportunity to understand molecular processes underlying "Hit-and-Run" carcinogenesis.

Keywords: gastric cancer, *Helicobacter pylori*, CagA, EPIYA motif, CM motif, Hit-and-Run carcinogenesis

Introduction

Gastric cancer is the fifth most common malignancy, with approximately 950,000 new cases registered each year, and the third leading cause of cancer-related deaths worldwide, accounting for more than 700,000 victims every year.[1)] It is the most common cancer in several geographic regions of the world, most notably East Asia. More than half of total gastric cancer patients are from East Asian countries such as Japan, China and Korea, and the incidence of gastric cancer in these countries is almost ten-times higher than that in the United States. Like other cancers, gastric cancer is a male-dominant malignancy and the incidence in men is almost twice higher than that in women.

According to Lauren's classification, gastric cancer is divided into two major types, intestinal type and diffuse type.[2),3)] Intestinal-type gastric cancer is well-differentiated, grows slowly and forms glands. This type of gastric cancer often arises via sequential pathological changes, starting from atrophic gastritis, and progression to intestinal metaplasia and then to dysplasia.[4)] Histopathologically, intestinal-type cancer shows irregular tubular structures, multiple lumens, and reduced stromal region.

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Abbreviations: AID: activation-induced cytidine deaminase; ASPP2: apoptosis-stimulating protein of p53 2; CagA: Cytotoxin-associated gene A; CBS: C-terminal binding sequence; CM: CagA multimerization; CM^{AmI}: Amerindian type-I CM; CM^{AmII}: Amerindian type-II CM; CM^{E}: East Asian CM; CM^{W}: Western CM; CSK: C-terminal Src kinase; EBV: Epstein-Barr virus; EMT: epithelial-to-mesenchymal transition; EPIYA: Glu-Pro-Ile-Tyr-Ala; GAB: Grb2-associated binder; *H. pylori*: *Helicobacter pylori*; IκB: Inhibitor of NF-κB; MALT: mucosa-associated lymphoid tissue; MARK: microtubule affinity-regulating kinase; NBS: N-terminal binding sequence; PAI: pathogenicity island; PAR1: Partitioning defective-1; PS: phosphatidylserine; RGD: Arg-Gly-Asp; SFK: Src family kinase; SHP1/2: SH2 domain-containing protein tyrosine phosphatase 1/2; TFSS: type IV secretion system.

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Diffuse-type gastric cancer spreads aggressively throughout the stomach without forming glands and rapidly metastasize to remote organs/tissues. Diffuse-type gastric cancer is comprised of poorly differentiated tumor cells and is characterized by the production of mucus. A morphological variation of diffuse-type cancer rapidly invades the muscles of the stomach wall with extensive fibrosis, making the stomach wall thick, hard, and rubbery. This subtype of diffuse gastric cancer is called scirrhous gastric cancer or linitis plastica,[5] the prognosis of which is extremely poor. Diffuse-type gastric cancer tends to arise at a younger age than intestinal type does. A small percentage of diffuse-type gastric cancer is of familial origin, caused by germline mutations in the *CDH1* gene encoding E-cadherin.[6] In hereditary diffuse gastric cancer (HDGC) cases, the tumor often develops at an age younger than 40 years.

Helicobacter pylori is a micro-aerophilic, spiral-shaped Gram-negative bacterium discovered in the epithelial lining of the stomach by Marshall and Warren.[7] Although *H. pylori* is not a commensal bacterium, it is estimated to infect approximately half of the entire human population. The acidic environment in the stomach does not allow the survival of viruses, bacteria and other microorganisms, but *H. pylori* has evolved to uniquely overcome this harsh environment. *H. pylori* secretes urease, an enzyme that converts urea into ammonia and bicarbonate to neutralize gastric acid, making the stomach a more hospitable place for *H. pylori*. Upon acquisition of the ability to survive, the stomach provides *H. pylori* with a special living niche. Host immune cells as well as antibodies that would normally recognize and attack invading bacteria are unable to reach *H. pylori* freely swimming in the mucus lining of the stomach. Instead, ineffective host immune responses continue to respond to the site of infection, where immune and epithelial cells die and release nutrients that feed the gastric pathogen. *H. pylori* infection is primarily acquired during childhood and the transmission occurs through an oral-oral or fecal-oral route primarily within families, particularly in the setting of poor sanitation and hygiene. In the majority of cases, colonized *H. pylori* persists in the stomach over the lifetime of the individual host unless eradicated with antibiotics. Just after its discovery, *H. pylori* was revealed to play a major etiological role in chronic atrophic gastritis and peptic ulcers. Subsequent clinico-epidemiological studies also indicated a close relationship between *H. pylori* infection and gastric cancer.[8–10] Based on these, the International Agency for Research on Cancer, World Health Organization (IARC/WHO) classified *H. pylori* as a group I carcinogen, a definite cause of cancer in humans, in 1994. Additional large-scale prospective cohort studies confirmed the association.[11,12] Studies on infection in rodents with *H. pylori* independently provided evidence for the role of the bacterial pathogen in the development of gastric cancer.[13–16] Both intestinal- and diffuse-types of gastric cancers are associated with *H. pylori* infection.[17] It is now well accepted that *H. pylori* is the single greatest risk factor for the development of gastric cancer, and the stomach pathogen is estimated to be the cause of ~75% of all gastric cancers.[18] The attributable risk, however, may be substantially underestimated in East Asian countries given that the frequency of gastric cancer without a history of *H. pylori* infection is incredibly low (1–3%) in this region.[19,20] Consistent with the etiologic role of *H. pylori* in the development of gastric cancer, large-scale intervention studies carried out in East Asian countries provided evidence that eradication of *H. pylori* by antibiotics is beneficial for preventing gastric cancer.[21–23] This review summarizes recent advances in the study of *H. pylori*-associated gastric cancer, particularly focusing on the role of the *H. pylori* CagA (Cytotoxin-associated gene A) protein, the first identified bacterial protein playing an active role in the development of human caner.

Type IV secretion system and CagA

Based on the presence or absence of *cagA*, a gene encoding the CagA protein, in its bacterial genome, *H. pylori* can be divided into *cagA*-positive and *cagA*-negative strains. The *cagA*-encoded CagA protein varies in size from 130 to 145 kilo Daltons (kDa) because of structural polymorphism in the carboxy-terminal (C-terminal) region (Fig. 1).[24,25] As expected from its name, CagA was originally considered to act as a bacterial cytotoxin.[26] However, subsequent study revealed that the acute cytotoxic activity of *H. pylori* was due to another bacterial toxin termed VacA (vacuolating toxin A).[27] The *cagA* gene is localized at one end of the *cag* pathogenicity island (*cag* PAI), a 40-kilobase genomic DNA segment that was acquired by a horizontal DNA transfer from an unknown donor.[28,29] Approximately 30–40% of *H. pylori* strains isolated in Western countries do not carry the *cag* PAI and thus are *cagA*-negative, whereas almost all of the *H. pylori* isolates from East Asian countries contain

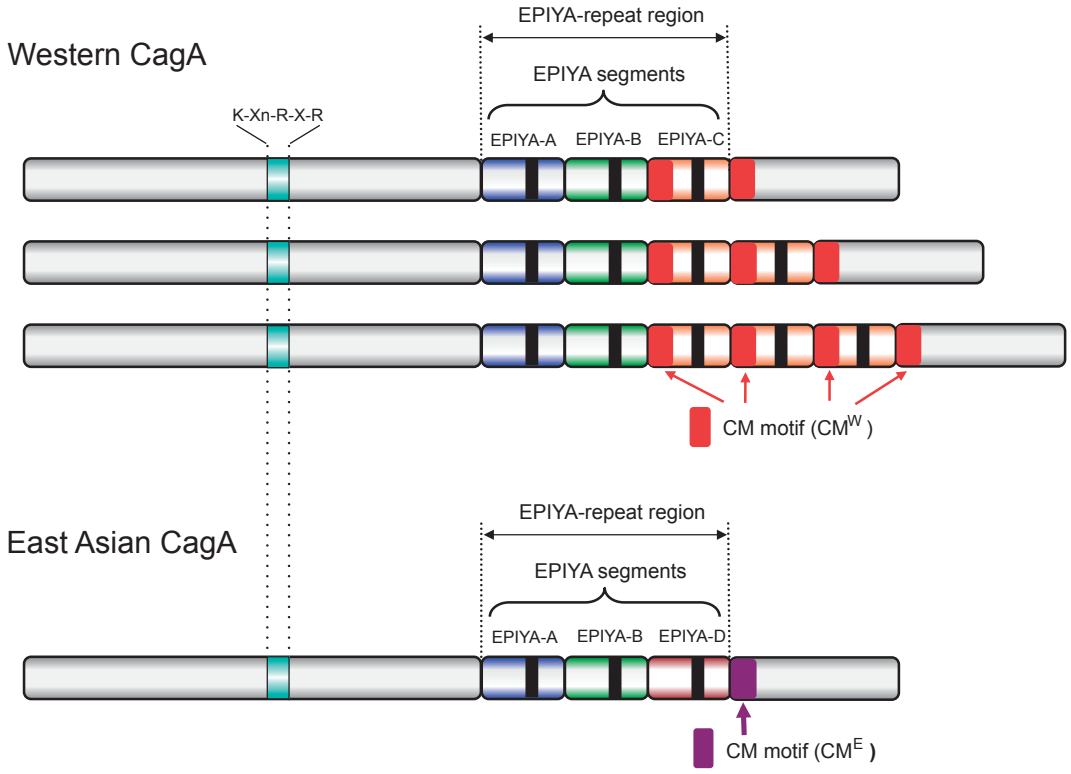

Fig. 1. Structural diversity of *H. pylori* CagA. The C-terminal EPIYA-repeat region of Western CagA comprises the EPIYA-A, EPIYA-B and a variable number (mostly 1–3) of EPIYA-C segments. The C-terminal EPIYA-repeat region of East Asian CagA comprises the EPIYA-A, EPIYA-B and EPIYA-D segments. Each of the EPIYA segments contains a single EPIYA tyrosine phosphorylation motif (shown as a black box), which is phosphorylated by kinases such as SFKs and c-Abl. East Asian CagA has a single CM (CM^E) motif immediately downstream of the EPIYA-D segment. Western CagA possesses at least two CM motifs, one in the EPIYA-C segment, which is unique to Western CagA (CM^W) and the other located distal to the last EPIYA-C segment (either CM^W or CM^E). The K-Xn-R-X-R motif in the central region is required for the binding of CagA with the membrane phospholipid, phosphatidylserine (PS).

the *cag* PAI and are thus *cagA*-positive.[30),31)] Depending on strains, the *cag* PAI contains 27–31 putative genes, including *cagA*. Among them, at least 18 genes encode proteins serving as building blocks of a type IV secretion system (TFSS),[32)] which form a syringe-like structure that is capable of delivering macromolecules across two bacterial membranes (outer and inner membranes).[32)] An association of *H. pylori* CagA with increased risk of gastric cancer was first reported in 1995.[33)] The importance of infection with *cagA*-positive *H. pylori* in the development of gastric cancer was subsequently consolidated by a number of epidemiological studies[34),35)] as well as animal infection experiments.[16),36)]

Mechanism of CagA delivery

Adhesion of *H. pylori* on the surface of gastric epithelial cells, mediated via numerous adhesins such as BabA/B, SabA, OipA, HopZ, and AlpA/B, initiates and facilitates the formation of the pilus-like TFSS syringe.[37)] Once stably attached to gastric epithelial cells, *H. pylori cagA*-positive strains initiate delivery of CagA into the cytoplasm of the host cells via the TFSS.[38)–42)] Formation of a conduit that connects the bacterium and host cytoplasm to deliver CagA is triggered by the interaction of CagL, a pilus component of the TFSS, with the host membrane β1-integrin in a manner that is dependent of the Arg-Gly-Asp (RGD) motif, the sequence motif that recognizes β1-integrin, present in CagL.[43)] Through complex formation, CagL activates integrin signaling that stimulates Src family kinase (SFK) activity, which in turn phosphorylates delivered CagA. β1-Integrin also interacts with other TFSS components such as CagI and CagY as well as CagA independently of the RGD motif.[44)] These Cag proteins may cooperatively bind to integrin molecules to stabilize TFSS-host cell interaction. Additionally, CagA presented on top of the TFSS pilus interacts with the membrane phospholipid phosphatidylserine (PS),

which is usually enriched in the inner membrane leaflet but is aberrantly exposed to the outer membrane leaflet upon direct contact with *H. pylori*, to facilitate delivery of CagA into the cells.[45] Since translocation of CagA is attenuated by treatment of host cells with hydroxymethylglutaryl (HMG)-CoA reductase inhibitors, statins, cholesterol enriched in membrane lipid rafts may also be involved in TFSS-mediated CagA delivery.[46),47] The requirement of multiple proteins as well as membrane lipids in the translocalization of CagA across the plasma membrane indicates that the process may not be a simple physical penetration of the TFSS pilus through the membrane but rather require complicated biophysical/biochemical events, molecular details of which warrant further investigation.

EPIYA motif and CM motif

Upon delivery into gastric epithelial cells, CagA is tethered to the inner leaflet of the plasma membrane via two distinct mechanisms depending on the status of cell polarity. In polarized epithelial cells, interaction of the central region of CagA, which contains multiple basic amino acids including those constituting the PS-binding K-Xn-R-X-R motif, plays an important role for the membrane association of CagA (Fig. 1).[45] In non-polarized cells, however, the C-terminal region is primarily responsible for the membrane tethering of CagA.[48] Membrane-localized CagA then undergoes tyrosine phosphorylation at the Glu-Pro-Ile-Tyr-Ala (EPIYA) tyrosine-phosphorylation motif, present in multiple numbers in the C-terminal polymorphic region (EPIYA-repeat region) of the protein,[49] by SFK members such as c-Src, Yes, Fyn, and/or Lyn,[50),51] which are expressed in gastric epithelial cells at variable levels, and by c-Abl.[52),53] The structural diversity in the C-terminal region of CagA is due to complicated recombination events within the genomic region encoding the C-terminal CagA.[54] Based on the sequences flanking the EPIYA motifs, four distinct EPIYA segments, EPIYA-A, -B, -C and -D, each of which contains a single EPIYA motif, have been identified (Fig. 1).[48),55),56] The EPIYA-repeat region of CagA from *H. pylori* circulating all over the world except East Asian countries is in an arrangement of EPIYA-A, EPIYA-B and EPIYA-C segments (termed Western CagA or ABC-type CagA). The EPIYA-C segment, composed of 34 amino-acid residues, variably multiplies (in most cases 1–3 times) in tandem among distinct Western CagA species. CagA from East Asian *H. pylori* isolates also possesses EPIYA-A and EPIYA-B segments but not the repeatable EPIYA-C segment. Instead, it has a distinct EPIYA-containing segment, termed EPIYA-D. Accordingly, the EPIYA-repeat region of East Asian *H. pylori*-specific CagA is in an arrangement of EPIYA-A, EPIYA-B and EPIYA-D segments (termed East Asian CagA or ABD-type CagA). Since a single CagA protein never carries both EPIYA-C and EPIYA-D segments, the presence of EPIYA-C is the hallmark of Western CagA, whereas the presence of EPIYA-D is the hallmark of East Asian CagA. In addition to these two major CagA isoforms, variable alignments of these EPIYA segments create further diversity among CagA isolates.[57),58] Tyrosine phosphorylation of these EPIYA segments by host kinases does not seem to be a simple stochastic process because SFK members selectively phosphorylate the EPIYA-C and EPIYA-D segments whereas c-Abl phosphorylates all of the EPIYA segments. Interestingly, in the delivered host cells, a single CagA protein appears to undergo tyrosine phosphorylation on 1 or 2 EPIYA segments but never on 3 or more EPIYA segments, with the preferred combination of phosphorylated EPIYA segments being EPIYA-A and EPIYA-C in Western CagA and EPIYA-B and EPIYA-D in East Asian CagA. Phosphorylation of CagA in the host cells may therefore be a sequential event, starting with EPIYA-C or EPIYA-D phosphorylation by SFKs at an initial stage of infection, followed by phosphorylation of EPIYA-A or EPIYA-B by c-Abl at a later stage of infection.[59]

In addition to the EPIYA motif, the C-terminal CagA region contains another repeatable sequence motif, originally designated as the CagA-multimerization (CM) motif as CagA can multimerize (dimerize) through the motif.[60] The CM motif, comprising 16 amino-acid residues, is located immediately distal to the last EPIYA segment (Fig. 1). Whereas the CM motif is highly conserved, there are 5 amino-acid alterations between East Asian and Western CagA species. From this variation, the CM motif of Western CagA is termed as CM^{W} and that of East Asian CagA is termed CM^{E}.[61] Remarkably, the amino-terminal (N-terminal) 16-amino-acid stretch that constitutes the 34-amino-aid EPIYA-C segment is identical to CM^{W}.[60] Accordingly, multiplication of the EPIYA-C segment also increases the number of CM motifs in Western CagA (Fig. 1).

EPIYA-dependent CagA functions

Expression of CagA in cultured gastric epithelial cells such as AGS cells, either by infection, trans-

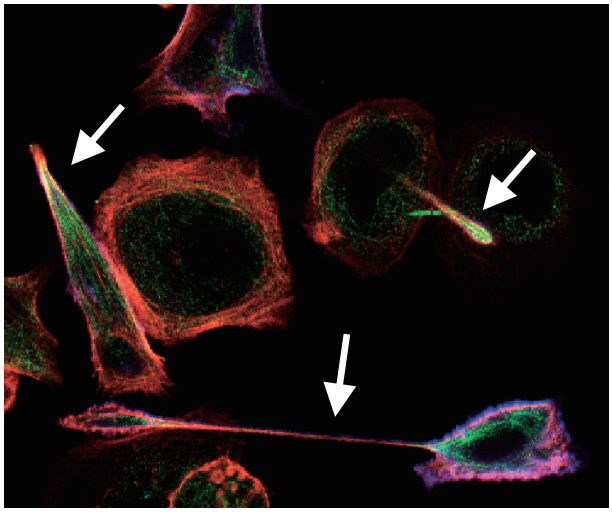

Fig. 2. The hummingbird phenotype induced by *H. pylori* CagA. Upon delivery into gastric epithelial cells, CagA induces an extremely elongated cell-shape known as the hummingbird phenotype (white arrows), which is concomitantly associated with elevated cell motility. Induction of the hummingbird phenotype requires tyrosine phosphorylation of CagA by host cell kinases.

fection, or transduction, induces a unique cell-morphological change termed the "hummingbird phenotype", which is characterized by an elongated cell shape with elevated cell motility and scattering (Fig. 2).[38] Induction of the hummingbird phenotype requires tyrosine phosphorylation of CagA in the cells.[49] Since the morphological change is reminiscent of the cellular change caused by treatment with hepatocyte growth factor (HGF), CagA was suggested to aberrantly activate intracellular signaling that is otherwise triggered by growth factor stimuli.[38] Such a role of CagA in signal perturbation was first provided by the finding that CagA specifically interacts with the Src homology 2 (SH2) domain-containing protein tyrosine phosphatase 2 (SHP2) (Fig. 3 and Table 1).[49] The CagA-SHP2 complex formation is mediated via the interaction between tyrosine-phosphorylated EPIYA-C or EPIYA-D segment and the SH2 domains of SHP2.[55]

In a physiological setting, SHP2 is required for full activation of the Ras-ERK mitogen-activated protein kinase (MAPK) pathway and is also involved in cell morphogenesis as well as cell motility.[62] SHP2 is catalytically activated by binding with membrane-associated scaffold/adaptors such as Grb2-associated binding (GAB) proteins. As in the case of CagA, SHP2 binding requires tyrosine phosphorylation of GAB, which is primarily mediated by receptor tyrosine kinases. This in turn raises the idea that CagA acts as a non-physiological scaffold/hub protein that mimics the function of GAB in mammalian cells.[63] This notion is supported by the results of an experiment showing that transgenically expressed CagA in photoreceptor cells in the *Drosophila* eye can substitute for the *Drosophila* Gab, Daughter of Sevenless (Dos), and this CagA activity requires the *Drosophila* SHP2, Corkscrew.[64] Expectedly, expression of CagA in gastric epithelial cells induces sustained ERK activation, which provokes promitogenic cellular response while morphologically inducing the hummingbird phenotype. Inhibition of CagA tyrosine phosphorylation, disruption of the CagA-SHP2 complex, knockdown of SHP2 expression by siRNA, or inhibition of ERK signaling abolishes the hummingbird phenotype by CagA.[49),65),66] In addition, CagA-deregulated SHP2 down-regulates the kinase activity of focal adhesion kinase (FAK) by dephosphorylating the activating phosphotyrosine residues (Tyr-369, -574, and -575), resulting in impaired focal adhesion turnover.[67] This CagA activity dampens cell-matrix interaction, which also promotes induction of the hummingbird phenotype. Deregulation of SHP2 by CagA may play an important role in terms of carcinogenesis because activating mutation of *PTPN11*, the gene encoding SHP2, has been found in a variety of human malignancies such as juvenile myelomonocytic leukemia (JMML), childhood myelodysplastic syndrome, B-cell acute lymphoblastic leukemia and acute myelocytic leukemia as well as several solid tumors.[68),69] Most of the reported *PTPN11* mutations are missense mutations in exons 3 and 8, which encode the N-SH2 domain and the phosphatase domain, respectively. Crystal structure analysis of SHP2 suggests that such mutations weaken the autoinhibitory intramolecular interaction that occurs between the N-SH2 domain and the C-terminal phosphatase domain and thereby constitutively activate the catalytic function of SHP2.[70] Binding of tyrosine-phosphorylated CagA to the SH2 domains also induces a conformational change in SHP2 that relieves the autoinhibitory composition, resulting in the aberrant activation of the SHP2 phosphatase activity.

Investigation of a new SHP2 substrate(s) potentially involved in CagA-mediated oncogenesis revealed parafibromin/Cdc73, a core component of the RNA polymerase II-associated factor (PAF) complex.[71] Upon tyrosine dephosphorylation by SHP2, parafibromin stably interacts with β-catenin, which activates Wnt target genes. Together with

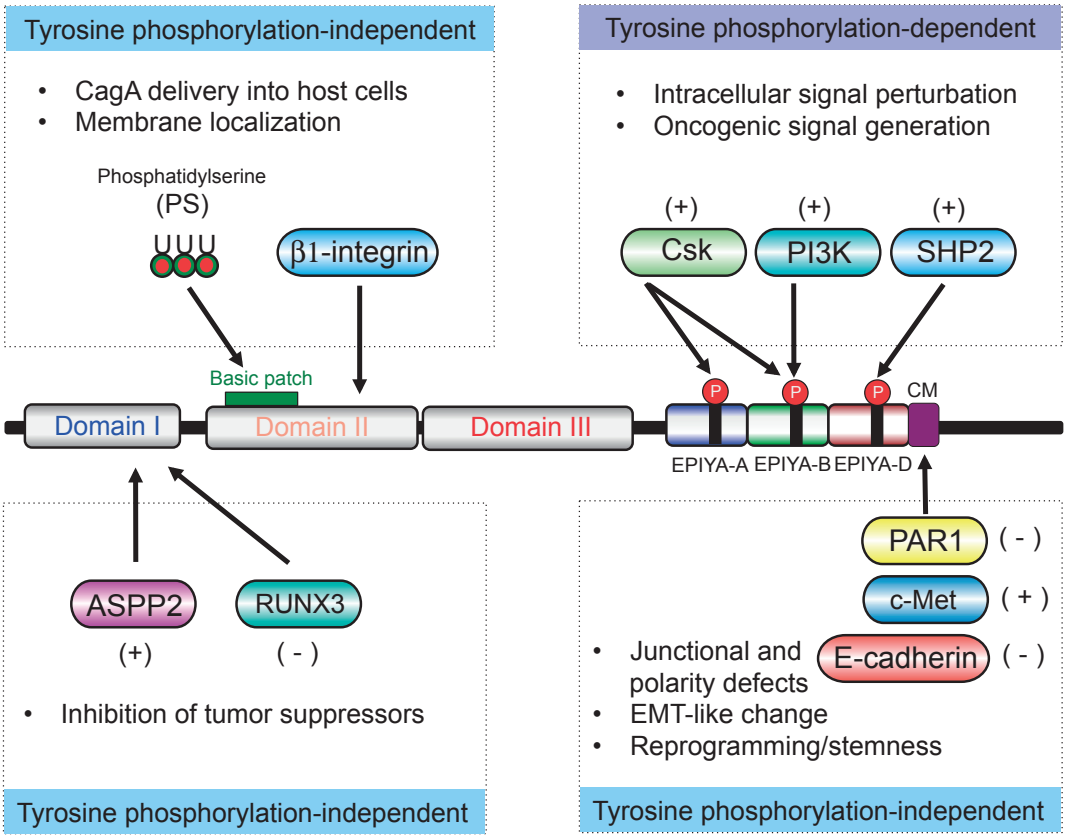

Fig. 3. Pathogenic scaffold function of CagA. CagA interacts with and thereby perturbs a number of host signal transducing molecules via tyrosine phosphorylated EPIYA motifs in its disordered C-terminal tail. CagA also binds to polarity regulating molecules via the C-terminal CM motifs, causing junctional and polarity defects. Domain I of the structured N-terminal CagA interacts with tumor suppressors, ASPP2 and RUNX1, resulting in inactivation of tumor suppressor functions. Domain II and/or Domain III of CagA are involved in CagA delivery into host cells and subsequent membrane localization. Through the interaction, CagA activates [indicated by (+)] or inactivates [indicated by (−)] the target proteins.

the Hedgehog (Hh) and Notch pathways, the Wnt pathway plays an essential role in the development, homeostasis, and pathogenesis of multicellular organisms. Parafibromin competitively interacts with β-catenin and Gli1, the transcriptional effector of the Hh pathway, thereby potentiating transactivation of Wnt- and Hh-target genes in a mutually exclusive manner.[72] Parafibromin also binds to the Notch intracellular domain (NICD), the transcriptional effector of the Notch pathway, enabling concerted activation of Wnt- and Notch-target genes. The transcriptional platform function of parafibromin is potentiated by tyrosine dephosphorylation, mediated by SHP2, while it is attenuated by tyrosine phosphorylation, mediated by protein tyrosine kinase 6 (PTK6) that is also known as breast tumor kinase (Brk). Thus, parafibromin integrates and converts signals conveyed by these morphogen pathways into appropriate transcriptional outputs in a tyrosine phosphorylation/dephosphorylation-regulated manner.[73] The role of the CagA/SHP2/parafibromin axis in gastric carcinogenesis warrants further investigation. Also notably, SHP2 forms a physical complex with transcriptional coactivators Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), targets of the cell density-sensing Hippo signal. Through the interaction, YAP/TAZ mediate nuclear translocalization of SHP2, which in turn dephosphorylates and activates parafibromin in the nucleus.[73]

The tyrosine-phosphorylated EPIYA-A or EPIYA-B segment serves as a docking site for the C-terminal Src kinase (CSK), a negative regulator of SFKs (Fig. 3 and Table 1).[74] The tyrosine-phosphorylated EPIYA-B segment also binds to the p85 subunit of phosphatidylinositol 3-kinase (PI3K) and activates the lipid kinase activity. Many of the Western CagA proteins carry a single nucleotide

Table 1. Host cell molecules that interact with CagA

Name of Molecule	Binding site on CagA	Pathobiological effect of the interaction
ASPP2	Domain I	Degradation and inactivation of p53
RUNX3	Domain I	Degradation and inactivation of RUNX3
Phosphatidylserine (PS)	Domain II (basic patch)	CagA delivery, Membrane tethering of CagA
β1-Integrin	Domain II	CagA delivery, Stimulation of SFKs and FAK
C-terminal Src kinase (CSK)	EPIpYA-A	Aberrant activation of CSK, Inhibition of SFK activity
	EPIpYA-B	
PI3K	EPIpYA-B	Deregulation of the PI3K-AKT pathway
SHP2	EPIpYA-C	Deregulation of the Ras-ERK pathway, Inhibition of FAK
	EPIpYA-D	
Grb2	EPIYA?	Deregulation of the Ras-ERK pathway
Crk	EPIpYA?	Loss of gastric epithelial cell adhesion
PAR1/MARK	CM	Disruption of tight junctions
		Loss of epithelial cell polarity
c-Met	CM	Deregulation of the Wnt pathway
		Enhanced cell motility
E-cadherin	CM	Disruption of adherens junctions
		Deregulation of the Wnt pathway
SHP1	N-terminal and C-terminal regions	Tyrosine dephosphorylation of CagA
TAK1	N-terminal and C-terminal regions	Activation of NF-κB activity

"EPIpYA" indicates a tyrosine-phosphorylated EPIYA segment.

polymorphism (SNP) in EPIYA-B that reduces CagA binding to PI3K.[75] SILAC (Stable Isotope Labeling by Amino Acids in Cell Culture)-based phosphoproteomics study showed that, in addition to the above-described SH2-containing proteins, the EPIYA-A peptide can bind to SHP1, the EPIYA-B peptide can bind to SHP1, Grb2, and SHP2, and the EPIYA-C peptide can bind to Grb2, Grb7, SHP1, and Ras-GTPase activating protein (GAP) in a manner that is dependent on EPIYA tyrosine phosphorylation, indicating the potential of tyrosine-phosphorylated EPIYA segments in promiscuously binding with numerous SH2 domains.[76] CagA is also capable of binding with Crk adaptor proteins (Crk-I, Crk-II, and Crk-L) in a tyrosine phosphorylation-dependent manner, though the responsible EPIYA segment remains unknown. Through the interaction, CagA disrupts adherens junctions by perturbing the adaptor function of Crk in cell signaling.[77] CagA binds to Grb2 via the EPIYA-containing region and thus aberrantly activates the Ras signaling pathway to stimulate abnormal cell proliferation.[78] However, the CagA-Grb2 interaction is independent of CagA tyrosine phosphorylation.

SHP1, the one and the only SHP2 homologue in mammals, is primarily expressed in hematopoietic cells, where it negatively regulates immune cell activation. Although less abundant, it is also present in the gastrointestinal linings. Like SHP2, SHP1 forms a physical complex with CagA in gastric epithelial cells. However, the interaction is independent of EPIYA tyrosine phosphorylation and potentiates the SHP1 phosphatase activity that dampens CagA action by dephosphorylating the EPIYA motifs. Accordingly, SHP1 is the long-sought host cell phosphatase that counteracts phosphorylation-dependent *H. pylori* CagA actions.[79]

EPIYA-independent CagA functions

The function of CagA as a pathogenic scaffold/hub in mammalian cells does not always require the EPIYA motif. The CM motif serves as a binding site for the polarity-regulating serine/threonine kinase Partitioning defective-1 (PAR1) (Fig. 3 and Table 1).[80),81] PAR1 was originally discovered as one of the PAR proteins (PAR1 to PAR6), which play essential roles in the establishment of cell polarity during *C. elegans* development. In mammals, PAR1 was independently identified as microtubule affinity-regulating kinase (MARK), which regulates the stability of microtubules by phosphorylating microtubule-associated proteins (MAPs) such as MAP1, MAP2, and tau.[82] Mammalian PAR1/MARK comprises four isoforms, PAR1a/MARK3, PAR1b/MARK2, PAR1c/MARK1, and PAR1d/MARK4. Of these, PAR1b is a predominant isoform in

epithelial cells. PAR1b distributes to the basolateral membrane of polarized epithelial monolayers, where it plays crucial roles in the establishment and maintenance of apical-basal polarity.[83),84)] Moreover, PAR1b inhibits a RhoA-specific guanine nucleotide exchange factor (GEF), GEF-H1, by phosphorylation to down-regulate RhoA and thereby inhibit actin stress fiber formation.[85)] Thus, the role of PAR1b in the cytoskeleton is not confined just to microtubule dynamics but also extends to filamentous actin (F-actin) rearrangements. CagA is capable of binding with all of the PAR1 isoforms,[86)] with the highest affinity to PAR1b, and the 15-amino-acid stretch within the 16-amino-acid CM motif, termed the MARK Kinase Inhibitor (MKI) peptide, directly occupies the kinase catalytic center of PAR1b to inhibit the kinase activity by mimicking host PAR1b substrates.[80),87)] Accordingly, the CagA-PAR1b interaction dampens the PAR1b kinase activity,[80),81)] which underlies junctional and polarity defects caused by delivery of CagA in the polarized epithelial layer,[88)] thereby making cells susceptible to oncogenic insults.[89)] For bacteria, the polarity defect might have an advantage as it enables them to replicate and grow in a larger area of epithelial cells.[90),91)] CagA is capable of homodimerization via the CM motif in cells.[60),92)] Given that PAR1 can exist as a homodimer in cells, CM-mediated CagA dimerization may represent juxtaposition of two CagA proteins by a PAR1 dimer. The CM motif also interacts with the activated HGF receptor c-Met, thereby potentiating c-Met-dependent motogenic response and enhancing c-Met-mediated activation of PI3K/Akt signaling (Fig. 3 and Table 1).[93),94)] This in turn leads to the stimulation of pro-oncogenic Wnt signaling and nuclear factor κB (NF-κB) signaling, which mediates cancer-promoting inflammatory responses.[95)] Additionally, CagA associates with the cytoplasmic domain of E-cadherin via the CM motif and thereby disrupts the E-cadherin/β-catenin complex, leading to aberrant activation of Wnt signaling (Fig. 3 and Table 1).[96)] Moreover, CagA is capable of forming a multimeric protein complex comprising E-cadherin, c-Met, and p120 catenin, though the functional relevance of the complex formation remains unknown.[97)] CagA also associates with glycogen synthase kinase (GSK)-3β via the C-terminal region containing the EPIYA and CM motifs. Through the complex formation, CagA sequesters GSK-3β to the insoluble fraction and thus potentiates Wnt activation by inhibiting the GSK-3β kinase activity.[98)]

In addition to the C-terminal region, the N-terminal region of CagA also interacts with several cellular proteins, which may additionally contribute to oncogenesis (Fig. 3 and Table 1). The N-terminal CagA region binds to the gastric tumor suppressor RUNX3 to promote proteasome-mediated degradation of RUNX3 by recruiting an unidentified E3 ubiquitin ligase.[99)] N-terminal CagA also enhances proteasome-dependent degradation of a p53 tumor suppressor by physically associating with apoptosis-stimulating protein of p53 2 (ASPP2).[100)] Also notably, CagA-activated Akt phosphorylates and activates human double minute 2 (HDM2) and ARF-binding protein 1 (ARF-BP1) E3 ligases that promote p53 degradation in conjunction with ASPP2. Since $p14^{ARF}$ is a negative regulator of both HDM2 and ARF-BP1, loss of the *ARF* gene locus in gastric epithelial cells may potentiate CagA-dependent p53 degradation.[101),102)] These findings collectively indicate that N-terminal CagA promotes survival of CagA-delivered cells by subverting pro-apoptotic signaling.

Structural basis of CagA action

CagA has a unique tertiary structure, consisting of a solid N-terminal region (70% of the entire CagA) and an intrinsically disordered C-terminal tail (30% of the entire CagA).[103),104)] The crystal structure of the N-terminal CagA region predicts a square plate-like shape with approximate dimensions of $110 \times 80 \times 55\,\text{Å}^3$, which comprises three discrete domains (Fig. 4). Domain I is the extreme N-terminal domain of CagA, having a small interacting surface area with Domain II but not with Domain III. As a result, Domain I is highly mobile and flexible. The intrinsically disordered proline-rich region of ASPP2 binds to the pocket formed by the three-helix bundle present in Domain I of CagA with high affinity that suffices co-crystallization.[105),106)] The P-P-X-Y motif of RUNX3 has been reported to interact with the WW domain of CagA.[107)] Although the presumed WW domain should be located in Domain I, there is no structural evidence for the presence of a functional WW domain in this CagA region. Domain II and Domain III comprise a protease-resistant structural CagA core. Domain II also contains a large anti-parallel β-sheet, with which CagA binds to β1-integrin for its delivery into the host cell.[104)] Domain II contains a basic patch, a cluster of basic residues containing those constituting the PS-binding K-Xn-R-X-R motif.[45)] The basic patch plays an important role in the interaction of CagA with PS.

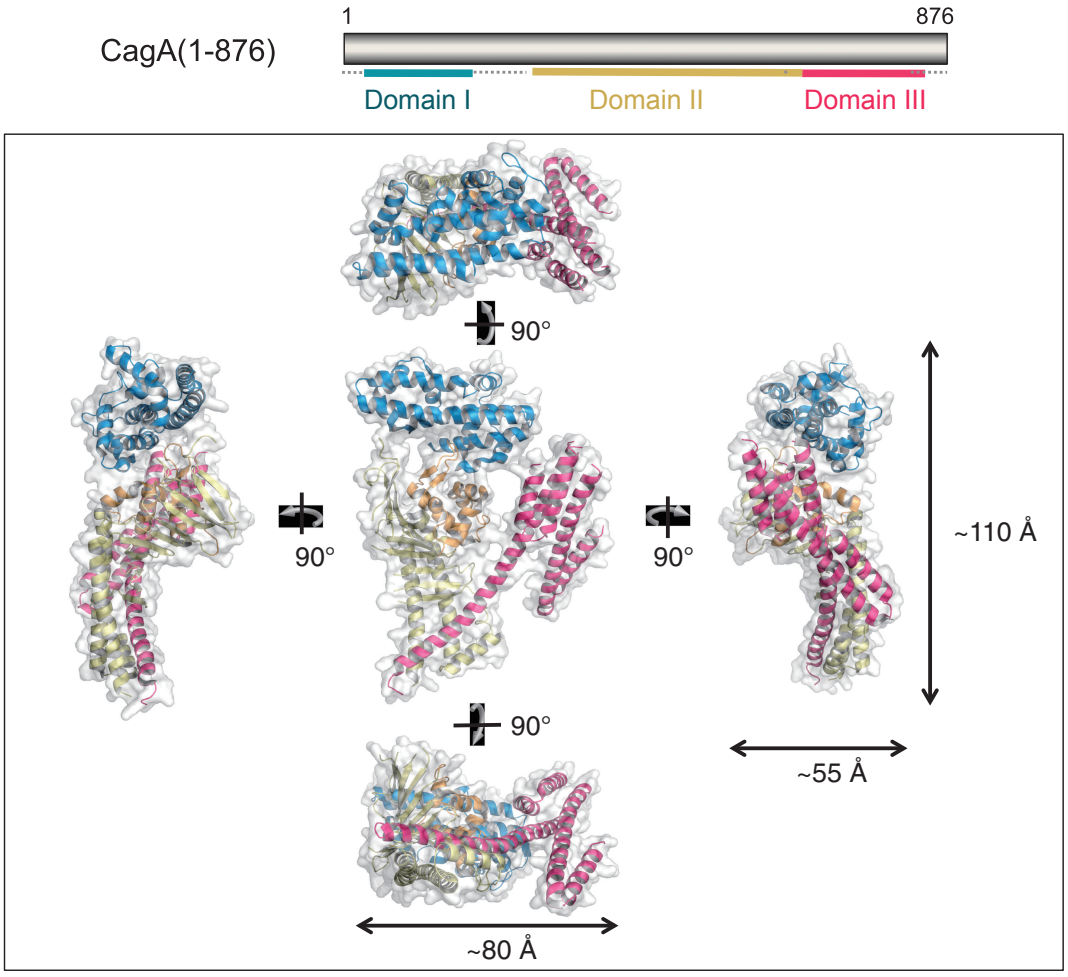

Fig. 4. Ribbon diagram of the crystal structure of the N-terminal structured region of CagA (residues 1-876 of CagA from *H. pylori* strain 26695). The CagA protein consists of structured N-terminal and disordered C-terminal regions. Folded N-terminal CagA displays a heretofore-unidentified structure with three distinct domains, Domain I (blue), Domain II (yellow), and Domain III (red). Domain I constitutes the N-terminus, while Domain II tethers CagA to the inner plasma membrane through electrostatic interaction between the basic patch and the acidic phosphatidylserine (PS) that is primarily distributed to the inner leaflet of the plasma membrane.

The N-terminal binding sequence (NBS) located within Domain III forms a four-helix bundle with the C-terminal binding sequence (CBS) located within the disordered C-terminal tail, creating a C-terminal lariat loop that strengthens interaction of CagA with PAR1 and SHP2 (Fig. 5).[103] Accordingly, the degree of the scaffold/hub function of CagA is fine-tuned by the intramolecular interaction of CagA. The highly diverged structure in the C-terminal CagA region may be explained by the intrinsically disordered nature of the EPIYA segments, which can be aligned in variable numbers and orders through homologous recombination at the DNA level with minimal structural constraints that hamper the CagA function.[108]

CagA as a bacterial protein that can promote carcinogenesis in metazoans

Despite accumulating *in vitro* evidence for the pro-oncogenic action of CagA, animal infection studies using mice or Mongolian gerbils have so far failed to convincingly demonstrate a causal role of CagA in oncogenesis. To challenge this important question, transgenic mice that systemically express wild-type or phosphorylation-resistant CagA, which does not have functional EPIYA motifs, were generated.[109] Mice expressing wild-type CagA showed gastric epithelial hyperplasia and some of them spontaneously developed gastric polyps as well as adenocarcinomas of the stomach and small

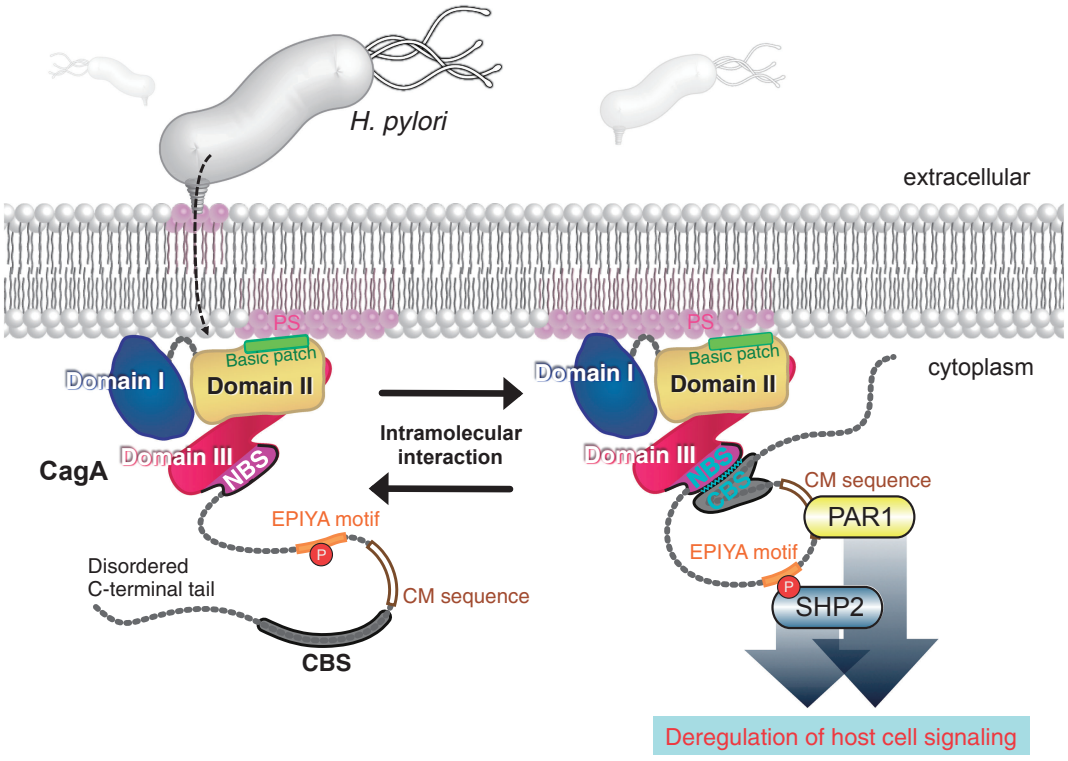

Fig. 5. Regulation of CagA activity by intramolecular interaction. CagA is delivered into gastric epithelial cells via the *H. pylori* type IV secretion system, where it acts as an oncogenic scaffold/hub. Intramolecular interaction, mediated between the N-terminal binding sequence (NBS) in Domain III and the C-terminal binding sequence (CBS) in the disordered C-terminal tail, potentiates binding capability of the tail with SHP2 and PAR1 via the EPIYA motif and CM motif, respectively, thereby strengthening the pro-oncogenic scaffold function of CagA.

intestine. The mice also developed leukocytosis that was associated with hypersensitivity to hematopoietic cytokines such as interleukin 3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) in bone-marrow cells, which may be due to hyperactivation of SHP2. Furthermore, a fraction of the transgenic mice spontaneously developed myeloid leukemias and B-cell lymphomas, hematological malignancies that are also caused by gain-of-function SHP2 mutations. In contrast, no pathological abnormalities were observed in transgenic mice expressing phosphorylation-resistant CagA. These results provide the first direct evidence for the role of CagA as a bacterium-derived protein that can stimulate carcinogenesis in mammals. The results also point to the importance of CagA tyrosine phosphorylation, which enables CagA to bind to and thereby deregulate SHP2, in the development of neoplasias. Likewise, transgenic expression of CagA in zebrafish increased the incidence of intestinal neoplasias caused by a mutation in the tumor suppressor gene *p53*,[110] providing additional *in vivo* evidence for the carcinogenic potential of CagA in animals. At the same time, the results of these transgenic experiments indicate the requirement of oncogenic collaboration between CagA and host cancer-associated genes in *in vivo* tumorigenesis.

It is well recognized that chronic inflammation promotes oncogenesis.[111] Infection with *H. pylori* *cagA*-positive strains provokes strong inflammatory responses in the stomach mucosa. However, there is no evidence for the presence of chronic inflammation in the gastrointestinal tract of CagA-transgenic mice.[109] Thus, to investigate the contribution of chronic inflammation in CagA-driven oncogenesis, CagA-transgenic mice were cyclically treated with a colitis inducer, dextran sulfate sodium (DSS).[112] Compared with control mice, DSS-induced colitis was markedly deteriorated in CagA-transgenic mice due to a substantial decrease in the cellular pool of IκB, which binds and sequesters NF-κB in the cytoplasm. Although the CagA-mediated IκB reduction did not automatically activate NF-κB, it lowered the threshold of NF-κB activation by inflammogenic insults. The incidence of colonic dysplasia was also increased in CagA-transgenic mice treated

with DSS. Thus, CagA exacerbates inflammation, whereas inflammation strengthens the pro-oncogenic potential of CagA. Through this functional interplay, CagA and inflammation reinforce each other in creating a downward spiral that instigates neoplastic transformation of epithelial cells.

H. pylori induces inflammation by utilizing multiple distinct mechanisms. Bacterial peptidoglycan can enter gastric epithelial cells via the TFSS, and it then stimulates the cytoplasmic pathogen recognition receptor nucleotide-binding oligomerization domain 1 (Nod1) to activate NF-κB for induction of inflammatory cytokines such as IL-1, IL-6, IL-8, and/or tumor necrosis factor α (TNFα).[113] *H. pylori* lipopolysaccharide (LPS), recognized by toll-like receptor 2 (TLR2) or TLR4, also activates NF-κB. Whereas CagA on its own does not seem to be a potent inflammogen, CagA delivered into host cells is capable of stimulating NF-κB-dependent transcription through multiple distinct mechanisms in addition to lowering the cellular pool of IκB. CagA triggers the PI3K/Akt/NF-κB signaling pathway by forming a complex with c-Met.[94] CagA also associates with TNF receptor associated factor 6 (TRAF6) and TGF-β-activated kinase 1 (TAK1) to induce TAK1 polyubiquitination, which in turn activates TAK1.[114] CagA-deregulated TAK1 is responsible for phosphorylation and stimulation of the IκB kinase (IKK) complex, leading to the activation of NF-κB and subsequent inflammatory response. However, it remains to be determined what extent of which CagA *per se* is involved in the activation of NF-κB in *H. pylori*-induced gastritis. *H. pylori cagA*-positive strains also induce hyperactivation of another proinflammatory transcription factor, signal transducer and activator of transcription 3 (STAT3). In the stomach mucosa, STAT3 is activated in response to stimuli by the IL-6 family of cytokines such as IL-6 and IL-11, resulting in induction of Janus kinase (JAK)/STAT3 signaling via the gp130 subunit of the IL-6 receptor. The gp130 subunit of the IL-6 receptor modulates the balance between SHP2/ERK signaling and JAK/STAT3 signaling. Expression of a gp130 mutant that lacks the SHP2 binding site in mice causes signal imbalance and thereby induces gastric inflammation and subsequent gastric cancer with high penetration.[115] CagA may also perturb the balance between SHP2/ERK signaling and JAK/STAT3 signaling by sequestrating SHP2 from gp130 by complex formation in gastric epithelial cells, resulting in the promotion of neoplastic transformation.[116]

Approximately 10% of gastric cancer cases are not only infected by *H. pylori* but also carry Epstein-Barr virus (EBV) in the tumor cells.[117] EBV-positive gastric cancer is noted for genome-wide CpG hypermethylation.[118] *In vitro* infection of gastric epithelial cells with EBV induces promoter hypermethylation of the SHP1-encoding *PTPN6* gene, which potentiates tyrosine phosphorylation-dependent CagA action via epigenetic downregulation of SHP1, the CagA phosphatase.[79] Clinical specimens of EBV-positive gastric cancers also exhibit *PTPN6* hypermethylation with reduced SHP1 expression. Augmented *H. pylori* CagA activity, via SHP1 inhibition, may therefore contribute to the development of EBV-positive gastric cancer.

In addition to gastric cancer, *cagA*-positive *H. pylori* is associated with the development of gastric mucosa-associated lymphoid tissue (MALT) lymphoma of B-cell origin. Most of the patients with MALT lymphoma are infected with *H. pylori cagA*-positive strains.[119] Eradication of *H. pylori* by antibiotics leads to regression of gastric MALT lymphoma in more than 75% of patients.[120] This clinical observation provides compelling evidence for a causal link between *cagA*-positive *H. pylori* and MALT lymphoma and raises the possibility that CagA is injected not only into gastric epithelial cells but also into B cells that have migrated to the gastric mucosa in response to *H. pylori* infection. Consistent with this, CagA is detectable in B cells that have infiltrated the stomach epithelial linings and also in tumor cells of MALT lymphoma.[121),122] CagA expressed in B cells by transfection gives rise to ERK activation, which results in the phosphorylation of Bcl2-associated death promoter (BAD) and thereby prevention of apoptosis of CagA-expressing cells.[123] CagA also inhibits apoptosis of delivered B cells by simultaneously perturbing the function of p53 and the JAK/STAT signaling pathway.[124] This CagA activity should promote accumulation of preneoplastic B cells by subverting their elimination though apoptosis, which may contribute to the development of MALT lymphoma.

CagA polymorphism in pro-oncogenic action

The CagA-SHP2 interaction, mediated by tyrosine-phosphorylated EPIYA-C or EPIYA-D, is one of key interactions through which CagA exerts its pro-oncogenic action. The sequence flanking the phosphotyrosine (pY) residue of the EPIYA-D segment perfectly matches the consensus high-affinity binding sequence for the SHP2 SH2 domains,

whereas the sequence flanking the pY residue of the EPIYA-C segment differs from the consensus sequence by a single amino acid at the pY+5 position.[55),56)] This finding indicates that East Asian CagA is more potent than Western CagA in terms of SHP2 perturbation. Indeed, clinical studies have revealed that gastric cancer is more closely associated with East Asian CagA-producing *H. pylori* strains than with Western CagA-producing *H. pylori* strains in geographical regions where two distinct strains co-circulate.[125)–127)] The notion is also supported by the observation that transgenic mice carrying East Asian CagA developed neoplastic lesions more frequently than did transgenic mice carrying Western CagA.[128)]

A unique feature associated with Western CagA is that the EPIYA-C segment tandem-duplicates with a variable number, mostly from one to three but possibly extending up to six.[129)] The proportions of Western CagA species containing one, two, and three EPIYA-C segments are approximately 60–70%, 20–30%, and <5%, respectively.[58)] In contrast to EPIYA-C, the EPIYA-D segment rarely duplicates in East Asian CagA species. As a result, East Asian CagA containing a single EPIYA-D segment binds to SHP2 more strongly than does Western CagA carrying a single EPIYA-C segment.[55)] Within Western CagA species, those having a greater number of EPIYA-C segments exhibit stronger ability to interact with SHP2.[57),130)] The results of a number of recent clinico-epidemiological studies have shown that infection with *H. pylori* strains carrying Western CagA with two or more EPIYA-C segments is more closely associated with the development of gastric cancer than is infection with *H. pylori* carrying CagA with a single EPIYA-C segment.[131)–136)] Since SHP2 binding is the only known CagA activity for which the magnitude is correlated with the number of EPIYA-C segments,[55),56)] the degree of CagA-SHP2 interaction may link the number of EPIYA-C segments with gastric cancer risk. Indeed, a quantitative study revealed that the strength of CagA-SHP2 binding is dramatically elevated by more than a hundredfold upon duplication of the CagA EPIYA-C segment from one to two (Fig. 6).[137)] This is most probably due to monomeric interaction *vs.* dimeric interaction of CagA with SHP2, which possesses two CagA-binding SH2 domains. The robust increase in the SHP2 binding activity of CagA by EPIYA-C duplication is also correlated with markedly enhanced cell invasion into the extracellular matrix, a malignant cellular trait caused by SHP2 deregulation.[138),139)] These observations provide a molecular basis for the role of multiple EPIYA-C segments as a distinct risk factor of gastric cancer.

As mentioned above, sequence polymorphism exists in the CM motif between Western CagA and East Asian CagA. Western CagA typically possesses 2–4 repeats of the CM^{W} motif as it usually contains 1–3 tandem repeats of the EPIYA-C segment that is followed by a single CM^{W} immediately distal to the last EPIYA-C repeat. East Asian CagA, in contrast, does not contain a CM motif in its EPIYA-D segment but carries a single CM^{E} immediately downstream of EPIYA-D.[60),61)] Interestingly, a fraction of Western CagA species carry CM^{E} rather than CM^{W} at the most distal CM motif.[140)] The CM motif, either CM^{W} or CM^{E}, serves as a binding site for PAR1. Non-canonical CM motifs have also been identified from other parts of the world such as Amerindian type-I CM (CM^{AmI}) and Amerindian type-II CM (CM^{AmII}) from indigenous tribes of the Amazon rainforest.[141)–144)] An increase in the number of CM^{W} motif in a single Western CagA synergistically augments its PAR1b binding, which is in proportional to the degrees of stress fiber formation and disruption of tight junction function (Fig. 7).[145)] For instance, a Western CagA containing 4 CM^{W} motifs exhibits a PAR1b-binding affinity that is more than 30-fold higher than that of a Western CagA containing a single CM^{W}. Also, a single CM^{E} shows a binding affinity to PAR1b that is comparable to that of two tandem CM^{W} motifs. In contrast, neither CM^{AmI} nor CM^{AmII} motifs bound to PAR1b, confirming that the binding of PAR1b to the Amerindian CagA proteins is extremely weak, if any.[144)] Accordingly, the CM polymorphism is a determinant for the magnitude of CagA-mediated perturbation of cytoskeletal systems, both microtubule-mediated and actin-mediated, which may influence clinical outcome of infection with *cagA*-positive *H. pylori* as recently described.[140)]

CagA, reprogramming, and genetic instability

H. pylori primarily targets mucus pit cells, terminally differentiated cells with a short life span of at most several days, for CagA injection. This raises a concern as to how such differentiated cells could be transformed by *H. pylori* CagA. One possible answer to this question is that CagA is capable of reprogramming and thereby de-differentiating fully terminated cells into tissue stem-like precursor cells. Indeed, CagA induces cell morphological change that resembles epithelial-to-mesenchymal transition (EMT) with properties in common with

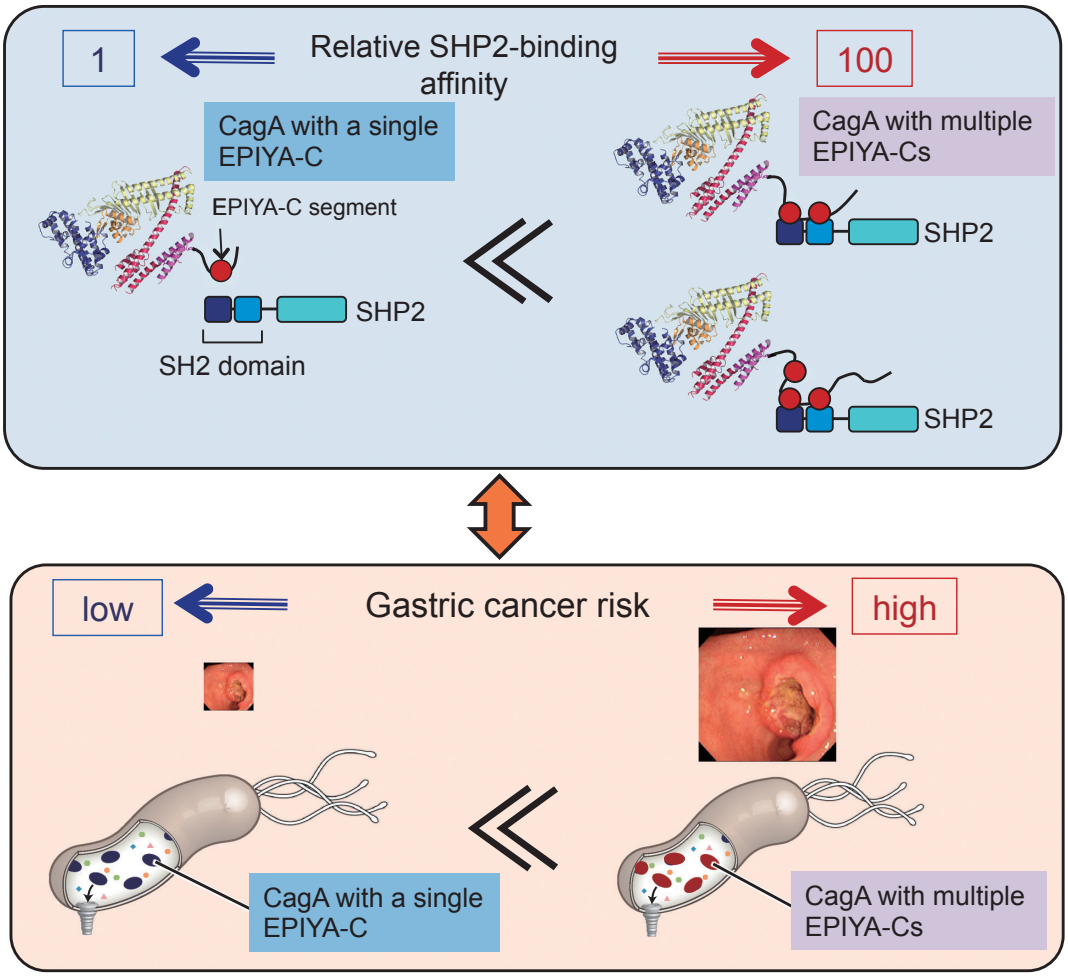

Fig. 6. EPIYA-C repeats and gastric cancer risk. Western CagA containing two or more EPIYA-C repeats (Type-II Western CagA) binds to both the N-SH2 and C-SH2 domain of SHP2. This bivalent interaction makes CagA-SHP2 complex formation extremely stable. On the other hand, interaction of CagA containing a single EPIYA-C segment (Type I Western CagA) with SHP2 is monovalent, making the CagA-SHP2 complex unstable. The decisive difference in SHP2-binding activity between CagA with a single EPIYA-C and CagA with multiple EPIYA-C repeats provides a molecular basis underlying an increased risk of gastric cancer in individuals infected with *H. pylori* carrying CagA with multiple EPIYA-C segments (Type-II Western CagA).

cancer stem cells.[146)–148)] Induction of EMT by CagA involves activation of Wnt signaling, triggered by disruption of E-cadherin/β-catenin interaction and/or c-Met activation.[94),96)] CagA also promotes EMT by inhibiting GSK-3β, which results in stabilization of Snail, a transcriptional repressor of E-cadherin.[98)] Mechanistically, CagA-mediated deregulation of Wnt signaling in gastric epithelial cells induces ectopic expression of the Caudal-related homeobox transcription factor Cdx1, which in turn transactivates reprogramming factors such as Sal-like protein 4 (SALL4) and Krüppel-like factor 5 (KLF5) to confer a stem-cell property in gastric epithelial cells. This CagA-mediated cell-fate reprogramming may underlie intestinal metaplasia, abnormal transdifferentiation of gastric epithelial cells to intestinal-like cells, that occurs in gastric mucosa that has been chronically infected with *cagA*-positive *H. pylori*.[149)] Although detailed mechanisms remain unknown, CagA expressed in gastric epithelial cells also provokes translocation of the nuclear factor of activated T cells (NFAT) transcription factor from the cytoplasm to the nucleus, thereby eliciting aberrant transactivation of NFAT-dependent genes.[150)] Recent studies demonstrated that activation of NFAT is required for successful reprogramming of somatic cells.[151)] Thus, CagA-mediated activation of NFAT may also be involved in the reprogramming process of gastric epithelial cells. In the meantime, a subpopulation of *H. pylori* colonizes

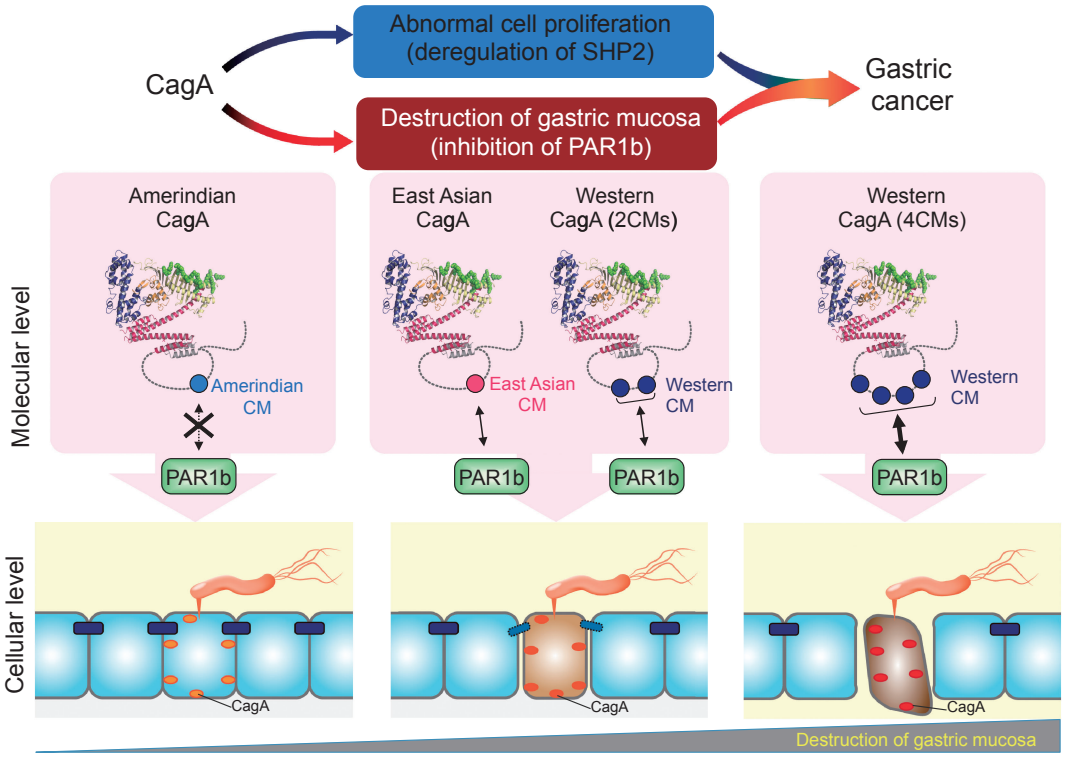

Fig. 7. Role of CM polymorphism in CagA-PAR1b interaction. CagA binds to PAR1b as well as other PAR1 family members via the C-terminal CM motif. Sequence diversity within the CM motif of CagA influences the strength of CagA-PAR1b interaction. PAR1b-binding affinity of East Asian CagA possessing one CM^E motif is equivalent to Western CagA containing two CM^W motifs. Western CagA possessing four CM^W motifs, though very rare (<5% of all Western CagA), exhibits an extremely strong binding to PAR1b.

the gastric gland base and directly interacts with the progenitor and stem cell compartments, giving rise to glandular hyperplasia with the expansion of Lgr5+ gastric stem cells in the antral glands in a CagA-dependent manner.[152] Thus, CagA may also be delivered into stem/progenitor cells in the stomach mucosa. Furthermore, CagA resists autophagic degradation in gastric epithelial cells expressing CD44v, a cell surface marker associated with cancer stem cells. This is because the autophagy pathway is suppressed in CD44v-positive stem-like cells due to their resistance to reactive oxygen species (ROS) through elevated levels of intracellular glutathione by CD44v.[153]

H. pylori CagA is an exogenous cancer-promoting protein that is injected intermittently into gastric epithelial cells by *H. pylori*. Once established, however, gastric cancer cells no longer require *H. pylori* and CagA for maintaining their transformed phenotype. Thus, gastric carcinogenesis should go through a "Hit-and-Run" process where dedifferentiated stem-like cells, led by CagA, eventually lose their CagA dependency.[154] Such a "Hit-and-Run" mechanism was originally proposed by Skinner for certain types of virus-associated cancers.[155] During the course of gastric cancer development, pro-oncogenic functions of CagA should be replaced or compensated by genetic and/or epigenetic alterations of the host genome. Obviously, this Hit-and-Run process will be accelerated in cells displaying genetic instability. Several studies have indicated that CagA on its own contributes to the disruption of genetic stability. CagA-mediated inactivation of PAR1, which perturbs microtubule stability, causes microtubule-based spindle dysfunction, leading to prophase/metaphase delay and subsequent misorientation of spindle formation.[156] As a result, chronic exposure of epithelial cells to CagA induces chromosomal instability leading to aneuploidy. Sustained activation of NF-κB in gastric epithelial cells infected with *H. pylori cagA*-positive strains induces aberrant expression of activation-induced cytidine deaminase (AID), a DNA editing enzyme that physiologically introduces somatic hypermutation in immunoglobulin variable regions in B cells. Ectopically expressed AID enhances accumulation of mutations in cancer-

related genes such as *p53* in gastric epithelial cells.[157] Infection with *H. pylori cagA*-positive strains is also associated with an increased level of H_2O_2 production, which causes oxidative DNA damage.[158] Additionally, CagA down-regulates the expression of Heme oxygenase-1 (HO-1), a potent anti-oxidant and anti-inflammatory molecule that cleaves the heme ring at the α methene bridge to form biliverdin, thereby enhancing genotoxic stress. A recent study demonstrated that *H. pylori* infection induces DNA double-strand breaks (DSBs).[159),160] However, since *H. pylori* mutants possessing functional TFSS but lacking the *cagA* gene efficiently induced DSBs in gastric epithelial cells, CagA is unlikely to be a major inducer of this type of DNA damage.

Pragmin, a host protein containing a functional EPIYA motif

A versatile EPIYA-dependent interaction of the bacterial CagA protein with a variety of SH2-containing mammalian proteins suggests that there might be a host EPIYA-containing protein(s) that functions as a scaffold molecule by utilizing a tyrosine-phosphorylated EPIYA motif. Evolutionally, CagA might have acquired the EPIYA motif by mimicking the function of such a host cell protein. Pragmin, also known as Sgk223, was originally identified as a downstream effector of Rnd2, a Rho family small GTPase, in neuronal cells.[161] Pragmin associates with Rnd2 and thereby stimulates RhoA to induce cell contraction, which then inhibits neurite outgrowth by nerve growth factor. Also, Pragmin possesses a pseudokinase domain in the C-terminal region. Pragmin is one of the few human proteins that contain the EPIYA motif, which undergoes tyrosine phosphorylation by SFKs or CSK.[162] In addition, CSK directly interacts with Pragmin via the tyrosine-phosphorylated EPIYA motif and this interaction potentiates the kinase activity of CSK. The phospho-EPIYA-dependent Pragmin-CSK interaction generates a positive feedback loop of CSK activation at focal adhesions and thereby induces cell-morphological transformation with elevated cell motility, deregulation of which provokes aberrant cell migration and invasion that contribute to oncogenesis.[163] Indeed, several studies have shown a functional link between Pragmin and oncogenesis. An increase in the level of Pragmin promotes invasion of advanced colon carcinoma cells.[164] Also, Pragmin is overexpressed in pancreatic ductal adenocarcinoma (PDAC) cells.[165] The pro-oncogenic activity of CagA might therefore involve deregulation of Pragmin. There are bacterial effectors other than CagA that also contain EPIYA or EPIYA-like tyrosine phosphorylation motifs. Eukaryotic proteins containing the EPIYA motif such as Pragmin may provide a clue to the evolution of such effector proteins in pathogenic bacteria.[108]

CagA and extragastric disorders

Recent epidemiological studies have suggested that infection with *H. pylori cagA*-positive strains is associated with coronary heart diseases[166),167] and ischemic stroke.[168] Preeclampsia, a pregnancy-associated disorder characterized by hypertension and proteinuria, is another vascular disease related to *cagA*-positive *H. pylori* infection.[169] These studies indicated that CagA may also contribute to the development of extragastric diseases. Many types of cells are known to release extracellular vesicles with unique biophysical and biochemical properties.[170] These vesicles are classified on the basis of their biogenesis; vesicles formed by exocytosis of multivesicular bodies are denoted as exosomes (with diameters ranging from 30 to 200 nm), while those budded directly from the plasma membrane are referred to as microvesicles (with diameters ranging from 100 to 1000 nm).[171] Extracellular vesicles are found in various biological fluids, including blood, urine, saliva, and breast milk, and they have been shown to play an important role in cell-to-cell communication through transport of informative constituents, including proteins, lipids, and microRNAs. CagA is detectable in serum-derived exosomes in patients infected with *cagA*-positive *H. pylori*.[172] Gastric epithelial cells expressing CagA also secrete exosomes containing CagA. The addition of purified CagA-containing exosomes to gastric epithelial cells induced the hummingbird phenotype (Fig. 2), indicating that the exosomes deliver functional CagA into cells. These observations raise the idea that exosomes secreted from CagA-injected gastric epithelial cells may enter into general circulation, delivering CagA to distant organs and tissues. CagA-containing exosomes might thus be involved in the development of extragastric disorders associated with *cagA*-positive *H. pylori* infection.

Conclusion and perspectives

Rapid progress in our understanding of *H. pylori* CagA has provided substantial insights into the molecular pathogenesis underlying the development of gastric cancer. CagA is the first identified bacterial protein functioning as a pro-oncogenic scaffold/hub

protein in animal cells. Given the Hit-and-Run mechanism of carcinogenesis conducted by the bacterial protein, a key question is the molecular event that converts cancer-predisposed gastric epithelial cells from CagA-dependent to CagA-independent. Oncogenic insults triggered by injected CagA in cultured epithelial cells provoke premature cell senescence and/or programmed cell death (apoptosis), primarily through the induction of cell cycle inhibitors such as $p21^{Cip1/WAF1}$.[65),96),148),150)] If such a cellular response, which serves as a fail-safe system against uncontrolled cell proliferation, is genetically or epigenetically perturbed in normal epithelial cells constituting the stomach mucosa, then evasion of host cells from oncogenic stress would become a key event that makes cells pass a "point of no return", after which cells acquire CagA independence. A molecular understanding of the transition process through the point of no return would require comprehensive genome-wide investigation of genetic/epigenetic alterations in gastric epithelial cells that have been exposed to CagA for variable periods with the use of next-generation sequencing technologies. Recently developed techniques for gastric organoids (gasteroids)[173)] as well as embryonic stem (ES) cell-derived stomach tissue[174)] should also provide a power tool for elucidating the molecular mechanisms underpinning acquisition of CagA independency. Assuming that established gastric cancer cells are still addicted to signaling pathways deregulated by CagA even after cells become CagA-independent, intracellular components conveying such signals will become critical molecular targets in the prevention and treatment of gastric cancer.

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Profile

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