ORIGINAL ARTICLE

Helicobacter pylori CagL dependent induction of gastrin expression via a novel $\alpha v\beta_5$-integrin—integrin linked kinase signalling complex

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ABSTRACT

Objective One of the most important hormones in the human stomach is the peptide gastrin. It is mainly required for the regulation of gastric pH but is also involved in growth and differentiation of gastric epithelial cells. In *Helicobacter pylori* infected patients, gastrin secretion can be upregulated by the pathogen, resulting in hypergastrinaemia. *H pylori* induced hypergastrinaemia is described as being a major risk factor for the development of gastric adenocarcinoma.

Design In this study, the upstream receptor complex and bacterial factors involved in *H pylori* induced gastrin gene expression were investigated, utilising gastric epithelial cells which were stably transfected with a human gastrin promoter luciferase reporter construct.

Results Integrin linked kinase (ILK) and integrin β5, but not integrin β1, played an important role in gastrin promoter activation. Interestingly, a novel CagL/integrin β5/ILK signalling complex was characterised as being important for *H pylori* induced gastrin expression. On interaction of *H pylori* with $\alpha v\beta_5$-integrin and ILK, the epidermal growth factor receptor (EGFR) → Raf → mitogen activated protein kinase kinase (MEK) → extracellular signal regulated kinase (Erk) downstream signalling cascade was identified which plays a central role in *H pylori* gastrin induction.

Conclusion The newly discovered recognition receptor complex could be a useful target in treating precancerous conditions triggered by *H pylori* induced hypergastrinaemia.

Significance of this study

To investigate the *H pylori* induced gastrin promoter activation, the bacterial ligand and its host receptor as well as the signalling pathway upstream of the promoter.

What is already known about this subject?

- *H pylori* induces the gastrin promoter.
- The induction proceeds in a CagA and VacA independent manner.
- The actual *H pylori* factor inducing gastrin is unknown.

What are the new findings?

- *H pylori* CagL is a novel ligand activating the gastrin promoter.
- CagL is interacting with αvβ5-integrin to activate gastrin expression via integrin linked kinase (ILK) signalling complex.
- The upstream signalling pathway of the gastrin promoter involves the epidermal growth factor receptor (EGFR) → Raf cascade leading to mitogen activated protein (MAP) kinase activation.

How might it impact on clinical practice in the foreseeable future?

- This newly discovered interaction partner CagL/β5-integrin could be a useful target in treating gastric precancerous conditions triggered by *H pylori* induced hypergastrinaemia.

INTRODUCTION

Helicobacter pylori is a gram negative, spiral shaped bacterium that colonises the gastric mucosa of approximately 50% of the world's population. This pathogen causes gastritis, peptic ulcer and, in some cases, gastric adenocarcinoma and mucosa associated lymphoid tissue lymphoma.[1 2] Gastric cancer is the fourth leading cancer causing death worldwide. In 1994, the WHO declared *H pylori* as a class I carcinogen based on epidemiological studies. *H pylori* strains harbouring the cytotoxin associated gene pathogenicity island (*cag*PAI) and certain alleles of the vacuolating cytotoxin (VacA) are more virulent than strains deficient in both virulence factors.[3–5] The *cag*PAI encodes a functional type IV secretion system (T4SS) to inject the effector protein CagA into host target cells[6] where it is phosphorylated by Src and Abl kinases.[7 8] Both phosphorylation dependent and independent signalling mechanisms of injected CagA have been described, such as activation of proinflammatory cytokines, cell proliferation, motility and elongation.[9] A recent study indicated that CagL, a VirB5 orthologue, is located at the tip of the T4SS pilus and interacts with the integrin $\alpha_5\beta_1$ receptor of the gastric epithelial cells via an Arg-Gly-Asp (RGD) motif.[6] CagL has been also shown to activate epidermal growth factor receptor (EGFR) and is essential for injection of CagA into gastric epithelial cells.[10]

Gastrin is one of the most important peptide hormones in the human stomach. It was first described in 1906 as a tissue factor which stimulates acid secretion in the corpus mucosa.[11] Gastrin is released into the gastric vasculature from

endocrine G cells in the antrum in response to food intake. Gastrin binds to cholecystokinin 2 receptors on enterochromaffin-like cells to stimulate histamine release and also binds to cholecystokinin 2 receptors on parietal cells to activate acid secretion in corpus tissue.[12 13] Histamine stimulates adjacent parietal cells through histamine 2 receptors and thus activates acid secretion also. It is well established that epidermal growth factor (EGF) binds to its receptor EGFR activating the gastrin promoter via the mitogen activated protein (MAP) kinase members, mitogen activated protein kinase kinase (MEK) and extracellular signal regulated kinase (ERK) 1/2.[14 15]

Studies in diverse animal models, usage of various human gastric cancer cell lines and the exploration of human biopsies established gastrin as a type of growth factor which affects proliferation, apoptosis, migration, invasion, tissue remodelling and angiogenesis both in vitro and in vivo.[16–23] INS-GAS and GAS-KO mice models are two animal models frequently used to investigate the physiological and pathological role of gastrin. INS-GAS mice which overexpress human amidated gastrin (GAS) under an insulin (INS) promoter exhibit initial gastric mucosal hypertrophy and excess gastric acid secretion. Gastrin deficient (GAS-KO) mice are achlorhydric due to a severe impairment of acid secretion. After 5 months, the INS-GAS mice display progressive changes in histology and physiology and after 20 months they are essentially achlorhydric and all animals develop gastric dysplasia. When these hypergastrinaemic mice were infected with *H pylori* expressing both CagA and VacA (*cagA*$^+$/*vacA*$^+$) they developed gastric adenocarcinoma after 7 months of infection.[24 25] However, in contrast with the INS-GAS mice, GAS-KO mice are characterised by reduced parietal cell numbers and are predisposed to bacterial colonisation and, therefore, are often overgrown by multiple bacterial species.[26–29] When these mice are infected with *cagA*$^+$/*vacA*$^+$ *H pylori*, there is an alteration in acid secretion, thought to be stimulated by a vagal response mechanism, but no increased risk of tumour development is observed after 6 months.[30] The absence of capacity for increased tumour development observed could be attributed to the lack of gastrin and its mitogenic function.

Mongolian gerbils are another useful model system to study *H pylori* induced gastric pathology. In our recently published study, gerbils were infected with the *H pylori* wild-type (WT) strain B8 which expresses a functional T4SS for injection and phosphorylation of CagA,[31] and an isogenic T4SS mutant strain B8Δ*cagY* (also called Δ*virB10*) which is not able to translocate the CagA into the host cells. The results revealed early (4–8 weeks) T4SS dependent inflammation only in WT infected animals that was followed by severe active and chronic gastritis, mucous gland metaplasia and dysplasia. Interestingly, WT infected animals exhibited hypochlorhydria after 16 weeks and hypergastrinaemia after 32 weeks post infection but none of the mutant infected mice did. To summarise these observations, we assert that the early proinflammatory responses (increase in interleukin (IL)-1β, interferon γ, tumour necrosis factor α, IL-6 and keratinocyte-derived cytokine) are major drivers for physiological changes such as hypochlorhydria and hypergastrinaemia.[32 33]

To date, the role of *H pylori* in the pathology of developing gastritis, disturbed acid and gastrin homeostasis was not well understood. Recently, it was shown that live *H pylori* are able to target the gastrin promoter through GC-rich DNA elements.[34] However, it is not clear which *H pylori* factor is responsible for activating the gastrin promoter and which downstream signalling cascades are involved. Hence in the present study, we investigated in detail the molecular mechanisms of *H pylori* induced gastrin expression. To explore the regulation of the human gastrin gene by *H pylori*, we used an in vitro approach with AGS gastric epithelial cells stably transfected with a human gastrin promoter luciferase reporter construct (GAS-Luc). Our main interests were to systematically identify the *H pylori* ligand, its binding partner on the cell surface as well as the signalling cascade involved in inducing the human gastrin promoter.

MATERIAL AND METHODS

Bacterial strains and culture conditions

H pylori P12, B8, 7.13, P1, 26695, J99, Tx30a, Ka125[35] and 1061[36] were grown on GC agar plates (Oxoid, Vienna, Austria) supplemented with horse serum (5%), vancomycin (10 μg/ml), trimethoprim (5 μg/ml) and nystatin (1 μg/ml) (serum plates) and incubated for 2–3 days in a microaerobic atmosphere (85% N_2, 10% CO_2, 5% O_2) at 37°C. *E coli* strain DH5α (BRL) was grown on Luria–Bertani (LB) agar plates under oxic conditions at 37°C. *Campylobacter jejuni* C64[37] was grown at 37°C on Columbia blood agar plates with sheep blood plus (Oxoid) under microaerobic conditions. Re-isolates out of gastric mucosa of Mongolian gerbils were verified with a positive urease (urea broth, Oxoid), oxidase (DrySlide, BBL), and catalase (3% hyperperoxid solution) activity and streptomycin resistance.

Materials, reagents and antibodies

Anti-$ß_2$-integrin (blocking, MAB1388Z), anti-α$vß_3$-integrin (blocking, MAB2023Z), anti-$ß_4$-integrin (blocking, MAB2058Z), anti-α$vβ_5$-integrin (blocking, MAB1961Z), anti-αV-integrin antibodies and cell culture inserts were purchased from Millipore (Schwalbach, Germany). Anti-IL1ß (neutralisation, I3642), anti-integrin linked kinase (ILK) and IL-8 were purchased from Sigma-Aldrich (Taufkirchen, Germany). Inhibitors ZM336372, PD98059, SB203580, Gefitinib and AG1478 were purchased from Merck (Vienna, Austria). The phospho-tyrosine specific antibody PY99, anti-Erk, anti-p38, anti-focal adhesion kinase (FAK) and anti-GAPDH were purchased from Santa Cruz (Heidelberg, Germany). For the experiments in figure 6C, the phospho specific antibodies anti-EGFR-PY-845, anti-Raf-PS-259, anti-MEK-PS-217/221 and anti-ERK-PT-202/PY-204 were purchased from NEB Cell Signalling (Frankfurt, Germany). Pro- and anti-inflammatory cytokines IL-1β, interferon γ, tumour necrosis factor α, IL-4 and IL-10 were purchased from ImmunoTools (Friesoythe, Germany). Antibodies against phospho-p38 and phospho-Erk were purchased from Cell Signalling. The antibody against CagA,[38] anti-$ß_1$-integrin (blocking, AIIB2), and the purified VacA[39] were kindly provided by the laboratory of Rainer Haas (Max-von-Pettenkofer Institute, Munich, Germany). Anti-$ß_7$-integrin antibody (blocking, 121004) was purchased from BioLegend (Fell, Germany). As western blot loading control, an anti-α-tubulin antibody (Upstate, Frankfurt, Germany) was used.

Construction of Δ*cagL* and Δ*cagY* mutants

Standard techniques were used for routine DNA manipulation, subcloning and plasmid construction, as previously described by Sambrook and Russell.[40] The Δ*cagY* mutant was constructed as previously described.[33] To produce a *H pylori* B8 Δ*cagL* mutant, the plasmid pSH6 was constructed. pSH6 carries a kanamycin cassette in a *Xba*I site between two adjacent nucleotide regions of about 1000 bp up and down stream of the *cagL* gene. The PCR products were generated by using genomic DNA from *H pylori* B8 strain applying the primers for 5′-*cagL* fragment: FP 5′-CGG AGC TCA GGT TCA GAC ATC TTG CTT GG-3′ and RP 5′-GCT CTA GAG CAT CTT CTT CAC CCA TTT C-3′ and 3′-*cagL* fragment: FP 5′-CCT CTA GAG CCA ATT TTG AAG CGA ATG AG-3′ and RP 5′-CGG AAT TCG ACA ACA CTT

GAG TGG TTT AAA AC-3′. The amplified products were cloned into the restriction sites *Sca*I and EcoRI of pBluescript SK+ (Stratagene, La Jolla, California, USA).

The resulting plasmid was transformed into *E coli* DH5α and the re-isolated plasmid (QIAprep Spin Miniprep Kit, Qiagen, Hilden, Germany) subjected to DNA sequencing to verify sequence integrity.

The deletion mutant was obtained by transforming the constructed plasmid pSH6 into the natural competent *H pylori* B8 cells kept in liquid culture medium (Brucella broth plus 10% fetal calf serum). The transformed bacteria were selected on serum plates supplemented with kanamycin. The P12*cagL*-RGD mutants were constructed as previously described.[41]

In vitro gastrin promoter stimulation assay

AM0 cells are AGS cells stably transfected with a plasmid containing 240 base pairs of the human gastrin promoter expressing the luciferase reporter (240 GasLuc).[42] AM4 cells express the 240 GasLuc construct with a 4 base pair mutation within the GC-rich element preventing EGF responsiveness (gERE) to the gastrin promoter.[43] AM4 cells equally treated served as negative controls. Both cell lines were cultured in RPMI 1640 (Gibco BRL, Darmstadt, Germany) supplemented with horse serum (10%) under standard conditions. Cells at 70% confluence and starved for 24 h in Nutrient Mixture F12 (HAM; Gibco) without supplements were stimulated with bacterial suspensions of WT and mutant strains at a multiplicity of infection of 100:1 (bacteria per cell) or purified VacA and CagL for 5 h. Cells were harvested in lysis buffer (14 g/l K_2HPO_4, 2.67 g/l KH_2PO_4, 0.74 g/l EDTA, 1 g/l Triton X-100, 1 nM DTT), and luciferase activity was measured (MicroLumat Plus LB 96 V; Berthold Technologies, Bad Wildbad, Germany) and normalised to total cell protein. Blocking antibodies and inhibitors were preincubated 45 min before bacterial stimulation. To generate metabolic inactive bacteria, pretreatment with 0.15% NaN_3 was carried out for 20 min.

Purification of recombinant CagL

To clone WT CagL for protein expression in *E coli*, a PCR product corresponding to amino acid residues 21–237 of the protein (minus the predicted signal sequence) was cloned into the pET-28a vector (Novagen, Darmstadt, Germany) and sequenced. The CagL-RGA mutant was constructed using the QuickChange mutagenesis kit (Novagen, Vienna, Austria). WT and RGA mutant CagL were overexpressed and purified by means of 6×His tag applying a standard protocol, as described previously.[6] In brief, *E coli* strain BL21 (DE3) harbouring the CagL vectors was grown in 5 ml of LB medium at 37°C overnight. Then 500 ml of fresh LB medium were added and shaken for about 3 h until OD_{600}=1. IPTG (1 mM) was added and the bacteria were grown for another 2 h to induce CagL protein expression. Bacterial pellets were harvested, pelleted by centrifugation and then resuspended in ice cold CW buffer (50 mM KH_2PO_4-K_2HPO_4, pH 7.5, 200 mM NaCl) supplemented with protease inhibitor cocktail (Roche, Dublin, Ireland). After sonication, CagL protein present in the inclusion bodies was solubilised in buffer LW (50 mM KH_2PO_4-K_2HPO_4, pH 7.5, 200 mM NaCl, 6 M guanidine hydrochloride) and refolded in ice cold refolding buffer (52 mM Tris HCl, pH 8.2, 20 mM NaCl, 834 µM KCl, 1.1 mM EDTA, 2.1 mM reduced glutathione, 210 µM oxidised glutathione). CagL proteins were then further purified through Talon resin affinity chromatography (BD Biosciences, Heidelberg, Germany) and gel filtration in buffer CW through Sephacryl S-200 (16/60) according to the suppliers' recommendation (Pharmacia Biosciences, Munich, Germany). CagL protein concentrations were measured by BCA protein assay (Pierce, Dublin, Ireland), typically yielding a total amount of about 1.5 mg CagL in 10 ml of buffer. Purification of CagL was judged to be of >95% homogeneity by SDS-PAGE/Coomassie Blue staining. The folded conformation of the purified CagL proteins was confirmed by circular dichroism.[6] No indication of post-translational modifications of purified CagL such as disulphide formation or methylation was detected.[10]

Binding of CagL to integrin $\alpha_v\beta_5$ determined by Biacore surface plasmon resonance

Purified integrin $\alpha_v\beta_5$ (Chemicon, Darmstadt, Germany) was immobilised by amino coupling to the surface of a CM5 carboxymethyl dextran sensorchip. The analysis was performed as described previously.[6] Data were processed using the Biaevaluation software (V.4.1).

In vitro assays of AGS cells with recombinant CagL

Assays for the interaction of AGS cells with purified CagL were performed according to procedures described previously.[10] Briefly, six well plates were coated overnight at 4°C with 100 µl of 50 µg/ml purified CagL or BSA (Sigma, Vienna, Austria) in buffer CW as control. Non-specific binding sites were then blocked with 5% bovine serum albumin (BSA) in buffer CW for 2 h at 37°C. The AGS cells were grown as described above, trypsinised and then treated with soybean trypsin inhibitor according to the protocol of the manufacturer (Sigma). After washing with phosphate buffered saline, 4×10^5 AGS cells in RPMI were added to the wells and incubated for 3 h. In this time period, AGS cells attached to immobilised CagL (but not BSA) and the cells were harvested with a cell scraper, pelleted by centrifugation and resolved in 1× SDS-PAGE buffer for western blot analyses.

Plasmid transfection

Low passage AGS cells with 40–50% confluency were transfected with either pSP65SR$^+$ or pSP65SR$^+$CagA and 240GasLuc for 24 h using Lipofectamine 2000 (Invitrogen, Darmstadt, Germany) and Genejuice Tranfection Reagent (Novagen), respectively, according to the manufacturer`s instructions. The CagA overexpression plasmid pSP65SR$^+$CagA contains the *cag*A gene of *H pylori* strain NCTC11637 which was cloned into basic vector pSP65SRα.[44]

RNA interference

Low passage cells with 20–30% confluency were transfected with 50 nM small interfering RNA (siRNA) using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's instructions. siRNA against FAK, ILK and control siRNA (with an equal GC content) were purchased from Invitrogen and (for figure 5D) from Santa Cruz. Three days after transfection, cells were harvested and subjected to immunoblotting and luciferase measurement.

RNA isolation and real time RT-PCR

Gastric epithelial cells were harvested 24 h post transfection with FAK or ILK siRNA in RLT buffer (Qiagen RNeasy Mini Kit) with 1% ß-mercaptoethanol. RNA isolation was performed as described in the Qiagen RNeasy Mini Kit protocol. Using the TaqMan reverse transcription reagents (Roche) with random primers according to the kit protocol, 1 µg mRNA was transcribed into cDNA. Oligonucleotide primers specific for FAK, ILK and the housekeeping gene 18S rRNA were applied for real time RT-PCR (ABI PRISM 7000, Applied Biosystems, Darmstadt, Germany). For amplification, the FastStart Universal SYBR

Green Master (ROX) kit (Roche) was used according to the manufacturer's instructions. All data were normalised with the corresponding 18S rRNA transcription level using a comparative delta Ct method.

Immunoblotting

Cells were harvested in lysis buffer (PhosSTOP (Roche) phosphatase inhibitor cocktail tablets resolved in DPBS (Gibco) by scraping). These cell lysates were boiled in Laemmli sample buffer for 10 min. Boiled samples were subjected to SDS-PAGE using a minigel apparatus (Bio-Rad, Munich, Germany) and blotted onto a PVDF membrane using a semi-dry blot system (Biotec Fischer, Reiskirchen, Germany). The membranes were blocked with either 5% milk powder or 3% BSA in TBS buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl) and incubated overnight with the respective primary antibody at 4°C followed by secondary antirabbit IgG or antimouse IgG conjugated HRP for 1 h at room temperature. Immunoreactive bands were detected by ECL and radiography.

Animals and infection experiments

Specific pathogen free Mongolian gerbils (n=20 females) were infected as previous described.[32] Animals were challenged orogastrically with approximately 10^9 *H pylori* suspended in Brucella broth. All experiments and procedures carried out were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and approved by the Regierung von Oberbayern (AZ 55.2-1-54-2531-78/05). The animals were sacrificed after 8 weeks of infection, the stomach opened along the greater curvature and the gastric tissue conserved in antrum and corpus separately.

Statistics

Data are presented as mean±SEM. The results (raw data) were statistically analysed using the Student t test with SigmaStat statistical software (***p≤0.001; **p≤0.01; *p≤0.05 were considered significant; #p>0.05 was considered not significant).

RESULTS

H pylori strains with functional T4SS specifically induce the gastrin promoter

Earlier reports showed that *H pylori* infected patients exhibited elevated serum gastrin concentrations relative to control populations.[45 46] In line with these observations, *H pylori* eradication in humans was accompanied by reduction of gastrin concentrations. To better understand how gastrin expression is influenced by *H pylori*, an in vitro gastrin promoter study was performed. We used AGS gastric epithelial cells that were stably transfected with a luciferase reporter construct regulated by the human gastrin WT gene promoter (AM0) to examine human gastrin expression. In the first set of experiments, the cells were stimulated with different bacteria and, subsequently, gastrin promoter transactivation was determined. As a positive control, EGF, a natural agonist for stimulation of gastrin gene expression,[47] showed elevated gastrin activation as expected (figure 1). Interestingly, clinical *H pylori* strains lacking the entire *cag*PAI (Tx30a, Ka125 and 1061) showed a significantly reduced gastrin promoter activation in contrast with all other applied WT strains expressing the *cag*PAI, such as B8, P12, 7.13 and P1. Two other *cag*PAI positive *H pylori* WT strains, 26695 and J99, also revealed gastrin promoter activation but at moderate levels. As negative controls, *C jejuni* and *E coli*, two bacterial species usually resident in the gastrointestinal tract, were unable to activate the gastrin promoter. Thus transcriptional activation of the gastrin promoter in vitro appears to be a specific feature of *H pylori* WT strains that express a functional T4SS in the *cag*PAI (figure 1).

Binding of *H pylori* strains is essential for gastrin promoter activation

H pylori causes persistent infection in the gastric mucosa, which is the major colonisation site for the bacterium. To explore whether bacterial contact with gastric epithelial cells is necessary to induce the gastrin promoter, filter inserts were used to prevent contact between the bacteria and the cells. In this experimental setting, WT *H pylori* were unable to induce promoter activation, suggesting that direct bacterial contact is necessary (figure 2A).

In a second step, we focused on the investigation of the *H pylori* ligand that binds to the epithelial cells inducing activation of the gastrin promoter. As binding of bacteria to epithelial cells is mainly mediated through its major adhesins BabA, SabA and AlpA/B,[48–50] we underlined the above observation by applying the corresponding adherence defective mutants. Therefore, *H pylori* J99 WT and isogenic single and double adhesin mutant strains were used to stimulate AM0 cells. No differences in gastrin promoter induction between the J99 WT and its isogenic mutant strains were observed (figure 2B). These results confirmed the above data that binding of *H pylori* via the major adhesins BabA, SabA and AlpA/B is not required to activate the gastrin signalling pathway. In agreement with a previous study,[34] a series of infection and transfection experiments showed that CagA or VacA are also not required for gastrin activation (see supplementary figure S1,[51 52] available online only).

Next, we investigated if vital and metabolically active *H pylori* are required for elevated gastrin promoter activity during infection. To answer this question, we stimulated AM0 cells with either heat killed or sonicated *H pylori* lysates. The results of these experiments showed that neither heat killed nor sonicates were able to induce gastrin activity (figure 2C). In the following experiment, we prepared metabolically inactive *H pylori* cells by preincubation with sodium azide (NaN_3) resulting in structurally intact but metabolically inactive bacteria. NaN_3 treated

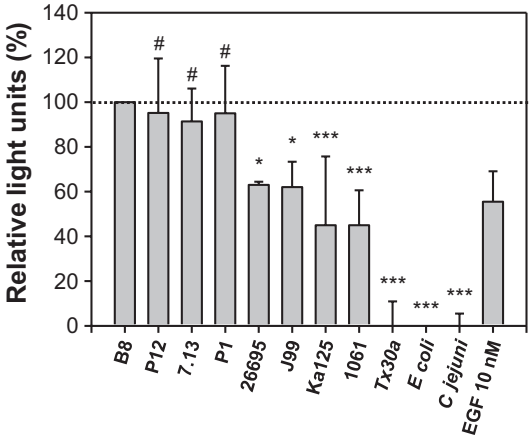

Figure 1 Gastrin promoter activation is *H pylori* strain specific. AM0 cells were stimulated with *H pylori* strains, *E coli* and *C jejuni*. After infection, cells were harvested and luciferase activity was determined. Luciferase activities in relation to *H pylori* B8 wild-type (represents 100%; interpolated broken line) were plotted. The mean values ± SEM of at least three independent experiments are shown. Statistical analysis (Student's t test) was applied using SigmaStat software. ***p≤0.001; *p≤0.05; #p>0.05. EGF, epidermal growth factor.

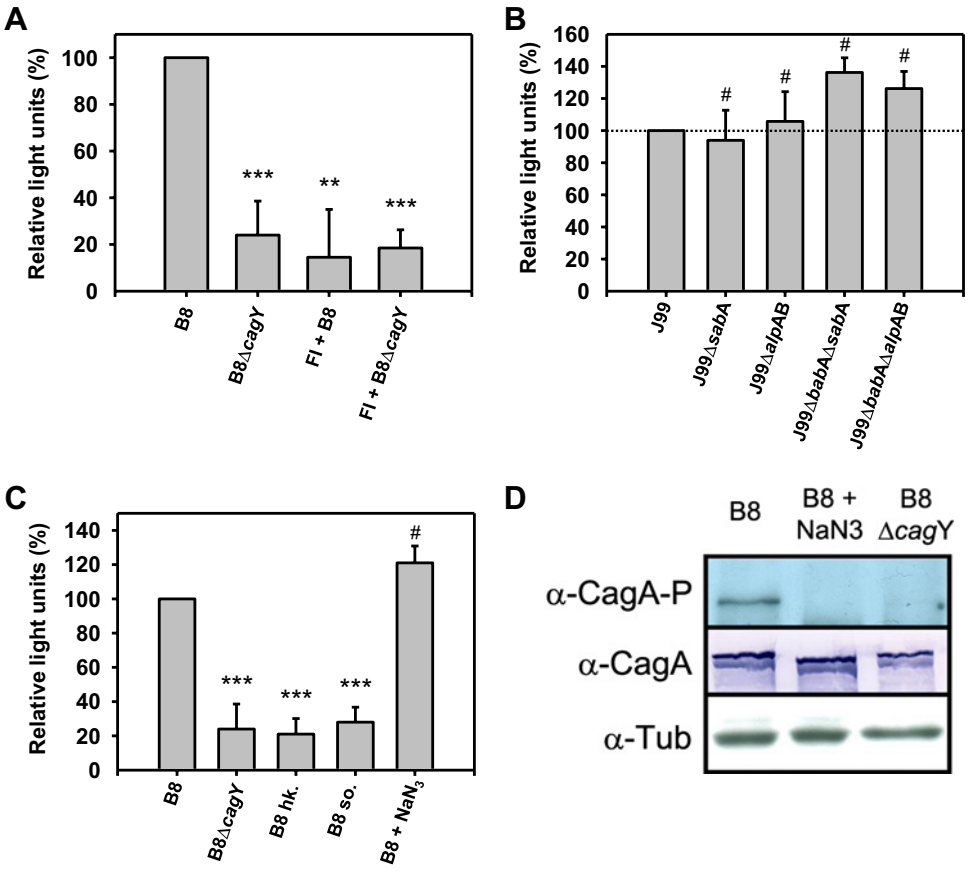

Figure 2 Contact of viable *H pylori* wild-type (WT) is essential for gastrin induction. (A) Filter inserts (FI) were placed in each well containing AM0 cells. *H pylori* B8 WT or B8Δ*cag*Y mutant strain were added. (B) AM0 cells were stimulated with *H pylori* J99 and isogenic adhesine mutant strains. (C) Heat killed (hk) and sonicated (so) *H pylori* B8 lysates, and NaN_3 treated, metabolic inactive were used to stimulate AM0 cells. (D) Western blot analyses of AM0 cells stimulated with NaN_3 treated, metabolic inactive *H pylori* strains were performed to detect translocated CagA using a phospho specific antibody. The B8Δ*cag*Y mutant strain served as a negative control and α-tubulin (Tub) as the loading control. Luciferase activities in relation to (A, C) *H pylori* B8 WT or (B) J99 (represents 100%; interpolated broken line) were plotted. The mean values ± SEM of at least three independent experiments are shown. Statistical analysis (Student's t test) was applied using SigmaStat software. ***p≤0.001; **p≤0.01; #p>0.05.

H pylori were no longer able to actively translocate CagA, as indicated by the absence of a phospho-CagA signal (figure 2D), but these metabolically inactive bacteria induced gastrin promoter activation similar to untreated *H pylori* WT (figure 2C). Taken together, these data indicate that a heat labil surface component of *H pylori* other than the well known adhesins BabA, SabA and AlpA/B or factors injected by the T4SS is sufficient to induce profound gastrin promoter activity. This indicates that a structural member of the *cag*PAI might be involved in promoter activation.

H pylori CagL induces the gastrin promoter

To identify the surface component responsible for gastrin promoter induction, we tested a series of *H pylori* mutants with defects in structural components of the T4SS, such as Δ*cagY* or Δ*cagL*. Interestingly, neither Δ*cagY* nor Δ*cagL* mutant was able to trigger gastrin promoter activation (figure 3A). The Δ*cagY* mutant was chosen because it can still produce CagL protein but does not produce T4SS pili for protein exposure.[53] This suggested that T4SS itself or a pilus exposed protein could trigger this signalling. A potential candidate was the CagL protein that decorates the T4SS pilus surface and makes contact with the integrin receptor.[6] Importantly, complementation of the Δ*cagL* mutant with the WT *cagL* gene restored promoter activity to WT levels, indicating that CagL plays a crucial role in gastrin promoter induction (figure 3A). In agreement with this conclusion, we also found that addition of recombinant CagL protein alone stimulated gastrin promoter profoundly (figure 3B).

H pylori CagL induces gastrin promoter activity via binding to $\alpha v\beta_5$-integrin

The above results suggest that CagL via interaction with the known integrin family member integrin $\alpha_5\beta_1$ may trigger

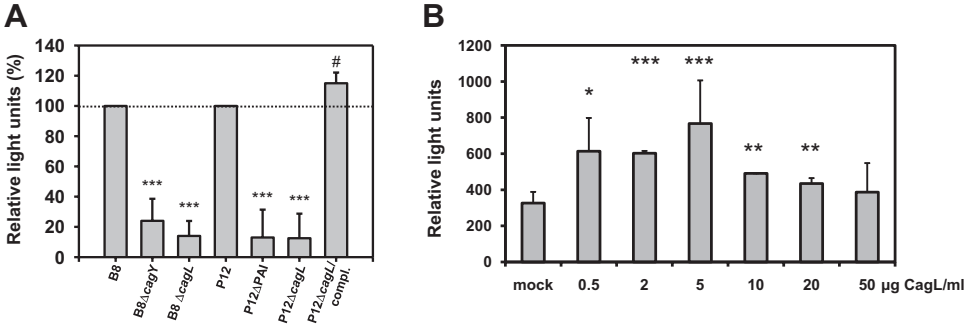

Figure 3 *H pylori* CagL is stimulating the gastrin promoter. (A) AM0 cells were stimulated with *H pylori* wild-type (WT) B8 and P12, and isogenic mutants B8Δ*cag*Y, B8Δ*cag*L, P12ΔPAI, P12Δ*cag*L and complemented Δ*cag*Lmutant strain. Luciferase activities in relation to *H pylori* WT (represents 100%; interpolated broken line) were plotted. (B) AGS cells transiently transfected with the gastrin promoter were stimulated with recombinant CagL (0.5—50 µg/ml), and luciferase activity presented. The mean values ± SEM of at least three independent experiments are shown. Statistical analysis (Student's t test) was applied using SigmaStat software. ***p≤0.001; **p≤0.01; *p≤0.05; #p>0.05.

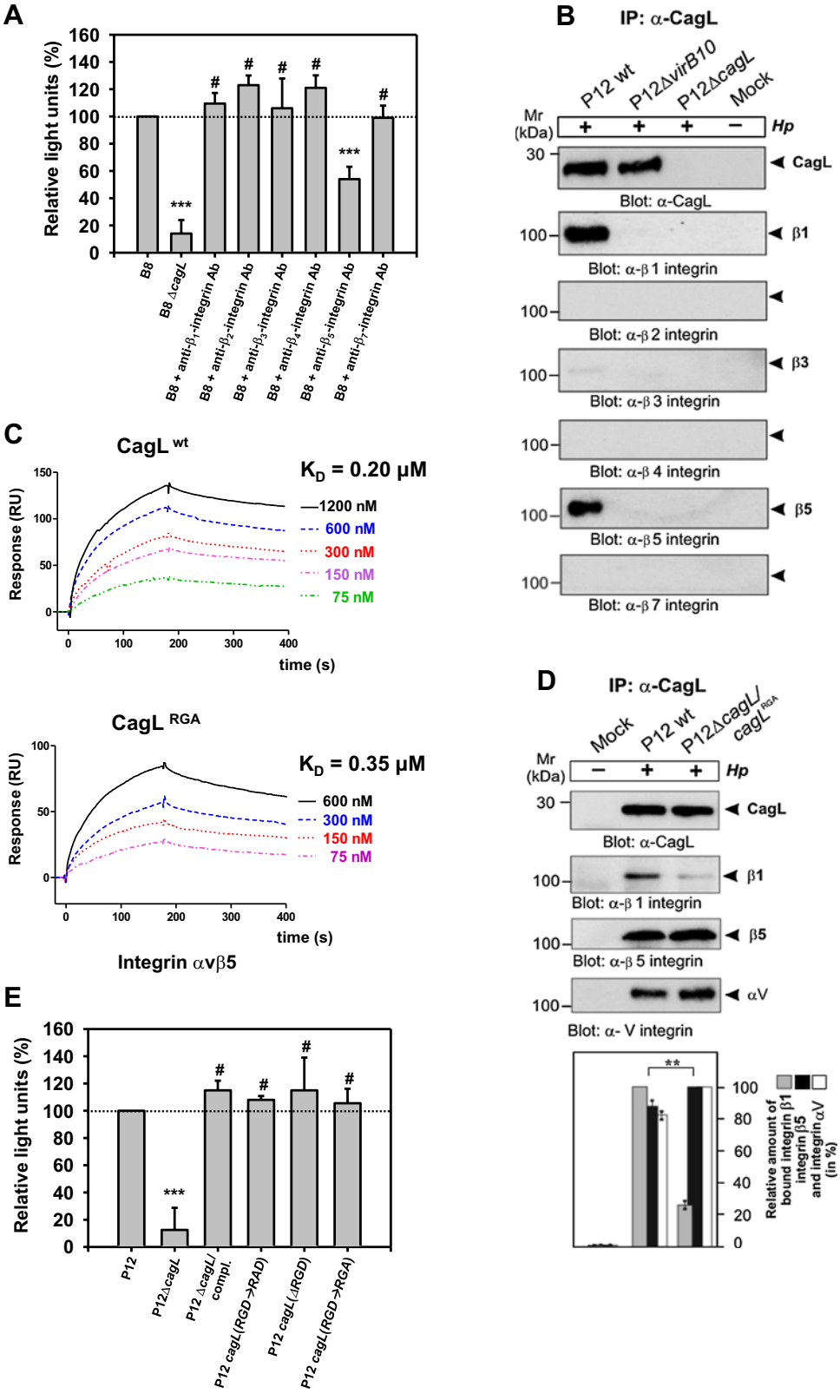

Figure 4 *H pylori* CagL binds to the epithelial cell via $\alpha v\beta_5$-integrin during infection. (A) AM0 cells were preincubated with anti-β-integrin blocking antibodies (Ab) (10 µg/ml) and stimulated with *H pylori* B8 strain. Luciferase activities in relation to *H pylori* B8 wild-type (WT) (represents 100%; interpolated broken line) were plotted. The mean values ± SEM of at least three independent experiments are shown. (B) Western blot detection of α-CagL immunoprecipitation of AGS cells stimulated with *H pylori* P12, Δ*virB10* (Δ*cagY*) and Δ*cagL* mutant applying α-CagL, β1, β2, β3, β4, β5 and β7 integrin antibodies. (C) Biacore in vitro binding analysis of recombinant WT CagL and $CagL^{RGA}$ mutant with αvβ5 integrin (K_D, dissociation constant). (D) Western blot detection of α-CagL immunoprecipitation of AGS cells stimulated with *H pylori* P12 and Δ*cagL* mutant complemented with $cagL^{RGA}$ applying α-CagL, α-β1, α-β5 and α-αV integrin antibody. Relative quantification of the bound integrin β1, β5 and αv (lower graph). (E) AM0 cells were stimulated with *H pylori* P12, isogenic P12Δ*cagL* mutant and complemented strain, and P12*cagL* strains with a deleted or mutated RGD motif. Luciferase activities in relation to *H pylori* B8 WT (represents 100%; interpolated broken line) were plotted. The mean values ± SEM of at least three independent experiments are shown. Statistical analysis (Student's t test) was applied using SigmaStat software. ***p≤0.001; **p≤0.01; #p>0.05.

downstream signalling leading to gastrin promoter activation. Interestingly, we found in infection experiments using AM0 cells preincubated with function blocking α-integrin antibodies (figure 4A) that binding to integrin $\alpha_5\beta_1$ is not required for gastrin activation. We therefore tested a number of other function blocking antibodies for major integrins expressed on AGS cells,[6] including β_2, β_3, β_4, β_5 and β_7 (figure 4A). Most strikingly, preincubation of cells with the α-$\alpha v\beta_5$-integrin blocking antibody significantly decreased gastrin promoter stimulation by more than 50% while the other antibodies had almost no effect (figure 4A). To make sure that CagL really interacted with integrin $\alpha_5\beta_1$ during infection, we infected cells with WT *H pylori*, a Δ*cagY* (Δ*virB10*) and Δ*cagL* mutant for 3 h followed by immunoprecipitation (IP) with an α-CagL antibody

(figure 4B). The western blot with α-CagL antibody showed that equal amounts of CagL protein were precipitated in each lane. Stripping and reprobing of the blot with α-integrin β_1 antibodies confirmed that β_1 integrin is indeed present in the IP complex (figure 4B). Surprisingly, we also detected strong signals for integrin member β_5, weakly for β_3, but not for β_2 or β_4 integrins. This suggests that CagL may trigger signalling via interaction with another receptor, $\alpha v\beta_5$-integrin, leading to gastrin promoter activation.

CagL interacts with both $\alpha_5\beta_1$-integrin and $\alpha v\beta_5$-integrin

We have recently shown in Biacore in vitro binding studies that recombinant WT CagL bound to $\alpha_5\beta_1$ integrin with high affinity (K_D of 0.09 μM) whereas the CagL^{RGA} mutant, in which the aspartate residue in the RGD motif was substituted by alanine, bound to $\alpha_5\beta_1$ integrin with fourfold less affinity.[6] To test if CagL can bind in the same manner to $\alpha v\beta_5$-integrin, we performed Biacore binding studies using the same settings. The results showed that WT CagL bound to purified $\alpha v\beta_5$-integrin with about the same affinity (K_D of 0.20 μM) compared with $\alpha_5\beta_1$ integrin (figure 4C). Interestingly, the CagL^{RGA} mutant bound $\alpha v\beta_5$-integrin with a similarly high affinity at a K_D of 0.35 μM (figure 4C), suggesting that the latter interaction proceeds in an RGD independent manner. In agreement with these data and previously reported findings,[6] we also observed that infection with *H pylori* expressing a CagL^{RGA} mutant bound less to integrin β_1. In contrast, in the same experiment, CagL^{RGA} bound about equally to integrin β_5 compared with WT CagL. To verify an RGD independent binding of CagL with integrin β_5, we infected cells with WT *H pylori* and a Δ*cag*L mutant complemented with a *cag*L^{RGA} mutated construct followed by IP with α-CagL antibodies (figure 4D). In each experiment, equal amounts of CagL were precipitated. The western blot analyses with α-integrin β_1 and β_5 antibodies clearly showed a significantly weaker and an equally intense band compared with the WT infected cells, respectively (figure 4D). Furthermore, reprobing of the IP with an α-αv integrin antibody revealed that the αv subunit is also present in the complex, as proposed (figure 4D).

CagL induces the gastrin promoter activity in an RGD independent manner

As the RGD motif seems to be dispensable for interaction of CagL with $\alpha v\beta_5$-integrin, we next aimed to investigate if this is also the case for signalling leading to gastrin promoter activation. For this purpose, we infected AM0 cells with WT *H pylori* and a series of additional RGD mutants (CagL^{RGA}, CagL^{RAD} or CagL$^{\Delta RGD}$). In agreement with the results above, we found that each of these mutants substantially induced gastrin promoter activation similar to that of WT bacteria (figure 4E). This further underlines the fact that CagL plays a crucial role in gastrin promoter activation in an RGD independent manner.

H pylori triggered host communication is mediated through integrin linked kinase

Integrins are specialised receptors in focal adhesions which link signalling from the extracellular matrix to intracellular factors. ILK and FAK are the most important focal adhesion components in mediating signals on β-integrin binding.[54] To analyse whether the binding of *H pylori* WT to $\alpha v\beta_5$-integrin triggers integrin signalling via FAK or ILK and eventually induces gastrin promoter stimulation, an RNA interference approach was established. siRNA was applied to achieve knockdown of endogenous ILK and FAK levels in AM0 cells. Real time PCR analyses revealed a knockdown efficiency of 80–90% on the mRNA level for FAK and ILK siRNA (figure 5A). A subsequent step was to confirm the knockdown of FAK and ILK on the protein level by western blotting. Following optimisation, after 72 h of siRNA transfection, neither ILK nor FAK proteins could be detected which verified an effective knockdown (figure 5B,D). AM0 cells were transfected with FAK or ILK siRNA for 72 h and then stimulated with *H pylori* WT. Our results established that downregulation of FAK did not abolish gastrin promoter activity but slightly enhanced it. However, all samples transfected with ILK siRNA showed a significant decrease in gastrin promoter induction of more than 50% (figure 5C). Therefore, we assume that induction of gastrin expression also involves ILK as a downstream signalling component.

EGFR/Raf/MAP/Erk signalling cascade involved in gastrin gene expression

Observations in various cell systems revealed that different intracellular MAP kinase signalling pathways (ERK, JNK and p38) downstream of membrane receptors may be involved in the transmission of gastrin inducing cellular effects.[55] In contrast with the signalling pathways activated by gastrin, the signal transduction leading to gastrin gene induction is not well characterised.[14] In a previous study it was suggested that *H pylori* involves MAP kinase using inhibitors targeting MEK1, JNK and p38.[34] To obtain a deeper insight into the regulation of the gastrin promoter by *H pylori*, we performed a detailed study of the upstream signalling.

Therefore, in this part of the study, we stimulated AM0 cells with *H pylori* WT, preincubated with kinase inhibitors against Raf-1 (ZM336372), MAP kinase MEK1 (PD98059) and p38 (SB203580). The data clearly revealed the involvement of the MAP kinase cascade (figure 6A). Western blot analysis showed that, compared with the *H pylori* Δ*cagY* mutant strain, *H pylori* WT activates the p38 signalling cascade to a higher extent. Erk signalling in AM0 cells was induced comparably in *H pylori* WT and *H pylori* B8Δ*cagY* mutant strains. The phosphorylation of Erk was no longer detectable when the cells were preincubated with the MEK1 kinase inhibitor PD98059 (figure 6B). Further western blot analysis of AGS cells stimulated with recombinant CagL protein clearly demonstrated activation of the signal cascade Raf→MEK→Erk (figure 6C) transactivating the gastrin promoter.

For several years it has been shown that EGF is a crucial signalling molecule in the stimulation of gastrin promoter activity via EGFR.[34] Whether *H pylori* induced transactivation of the gastrin promoter involves EGFR activation was investigated in this part of the study. Western blot analysis of CagL stimulated cells with α-phospho EGFR antibodies revealed EGFR as a candidate upstream receptor for the signal cascade involved in gastrin promoter activation (figure 6C). To verify the involvement of the EGFR in *H pylori* WT induced gastrin induction, we preincubated AM0 cells with Gefitinib and AG1478, tyrosine kinase inhibitors of the EGFR family resulting in significantly reduced luciferase activity (figure 6D). In parallel, we utilised a neutralising α-EGF antibody to bind free EGF which would also stimulate the gastrin promoter via EGFR. However, *H pylori* WT strain was still able to stimulate the gastrin promoter, independently of EGF (figure 6D). These data suggest that the *H pylori* induced gastrin promoter activation involves the phosphorylation of the EGF receptor independent of its classical EGF stimuli.

Furthermore, we analysed the impact of ILK siRNA treatment on EGFR–MAP kinase signalling in infected cells.

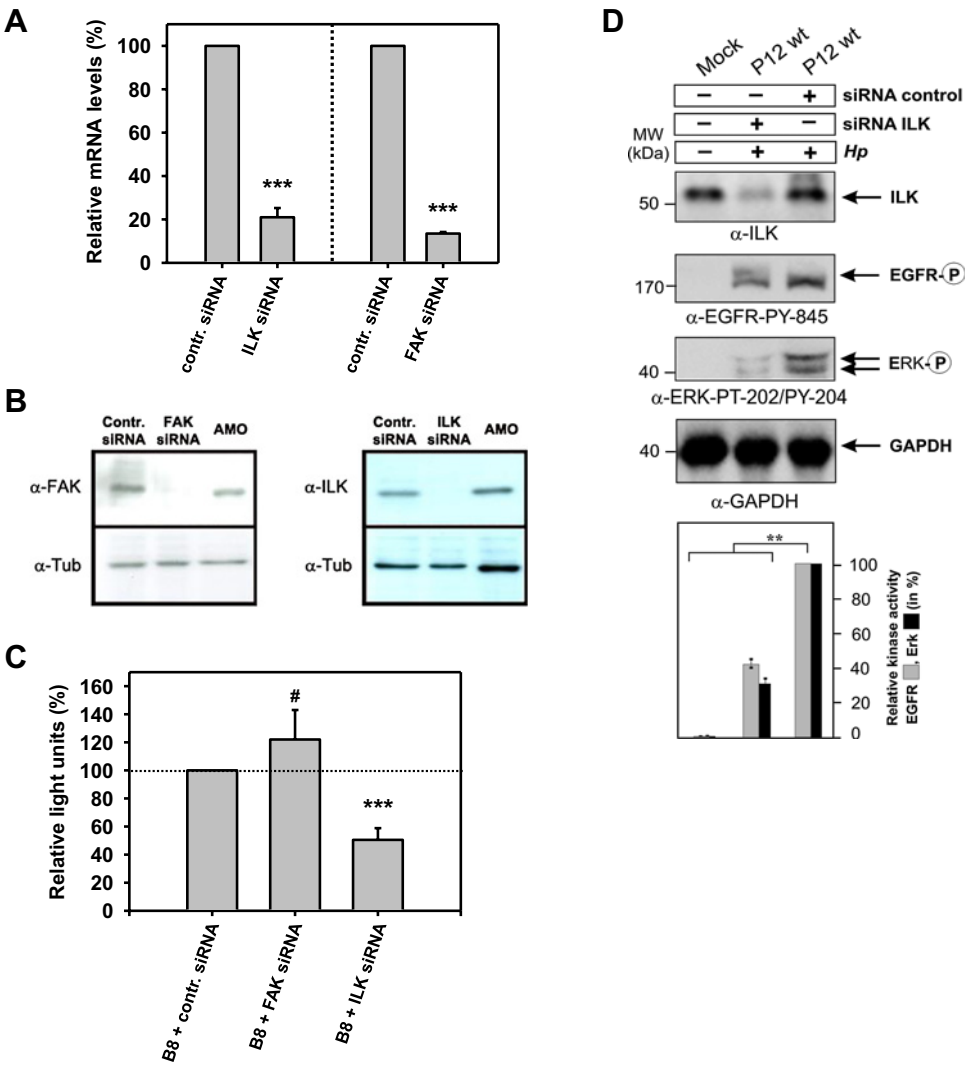

Figure 5 Integrin linked kinase (ILK) is involved in *H pylori* induced gastrin gene expression. (A) AM0 cells were transfected with either ILK small interfering RNA (siRNA 50 nM) or focal adhesion kinase (FAK) siRNA (50 nM) for 24 h. RNA was isolated and RT-PCR was performed. Control siRNA (50 nM) with the same GC content as the ILK siRNA or FAK siRNA was used as knockdown negative control (represents 100%). (B) Western blot analyses were performed to confirm ILK or FAK silencing on protein level. α-tubulin (Tub) served as a loading control. (C) AM0 cells were transfected with control siRNA (50 nM), FAK siRNA (50 nM) and ILK siRNA (50 nM) for 72 h and subsequently stimulated with *H pylori* B8 strain. Luciferase activities in relation to *H pylori* B8 wild-type (WT) transfected with control siRNA (represents 100%; interpolated broken line) were plotted. (D) Western blot analyses of AGS cells preincubated with control siRNA or ILK siRNA and stimulated with *H pylori* P12 were performed to detect ILK silencing, activation of epidermal growth factor receptor (EGFR) and extracellular signal regulated kinase (ERK), and loading control GAPDH using specific antibodies. Relative quantification of expressed phosphorylated EGFR and ERK (lower graph). The mean values ± SEM of at least three independent experiments are shown. Statistical analysis (Student's t test) was applied using SigmaStat software. ***p≤0.001; **p≤0.01; #p≥0.05.

Therefore, AGS cells were pretreated with ILK siRNA and then stimulated with *H pylori* WT. Activated EGFR and Erk were significantly reduced after ILK silencing. These data indicate that EGFR activation as well as MAP kinase signalling are linked to CagL binding to αvβ5-integrin and ILK activation (figure 5D).

WT *H pylori* but not Δ*cagL* mutant induces a severe antral gastritis in Mongolian gerbils

To investigate the newly discovered functional role of CagL in vivo, we used the well established Mongolian gerbil model for infection studies. For this purpose, Mongolian gerbils were orogastrically challenged with a suspension of *H pylori* WT strain B8 or an isogenic Δ*cag*L mutant. Eight weeks after infection the animals were sacrificed and longitudinal sections of antral and corpus mucosa were prepared for haematoxylin–eosin staining. The stained gastric sections of non-infected, WT and Δ*cag*L mutant infected animals were used for grading the inflammation (figure 7A–C). In this study, we obtained a re-isolation rate of 100% for each strain (data not shown). The gerbil adapted *H pylori* WT strain B8 induced severe and moderate inflammation in antral and corpus mucosa after 8 weeks of infection, respectively (figure 7B). In contrast, Mongolian gerbils challenged with an isogenic Δ*cag*L mutant strain revealed only a moderate to severe inflammation in the antrum but not in the corpus mucosa (figure 7C). These data are consistent with our previous observations of animals infected with a *H pylori* B8Δ*cag*Y mutant that only reveal a proinflammatory response in the antral mucosa, after 8 weeks of infection, whereas animals infected with *H pylori* WT, carrying a intact T4SS, were able to induce a corpus dominant gastritis after only 8 weeks of infection, resulting in a significant hypochlorhydria and hypergastrinaemia after 16 and 32 weeks of infection, respectively. This indicates a role for CagL in early proinflammatory responses as major drivers for such physiological changes, as reported previously.[32 33]

DISCUSSION

Secreted gastrin is a crucial hormone in mammals and regulates not only the pH of the stomach but also the growth and differentiation of gastric epithelial cells.[56] *H pylori* induced hypergastrinaemia was postulated to be a major risk factor promoting the development of gastric cancer.[24 32 33 57] To date the mechanism by which *H pylori* induces the gastrin signalling pathway is poorly understood. To elucidate how *H pylori* induces gastrin gene expression, an in vitro promoter study was performed. We report here the identification of a bacterial factor, CagL, which can activate a novel integrin linked signalling cascade to induce gastrin secretion as recombinant protein in vitro and during infection in vivo.

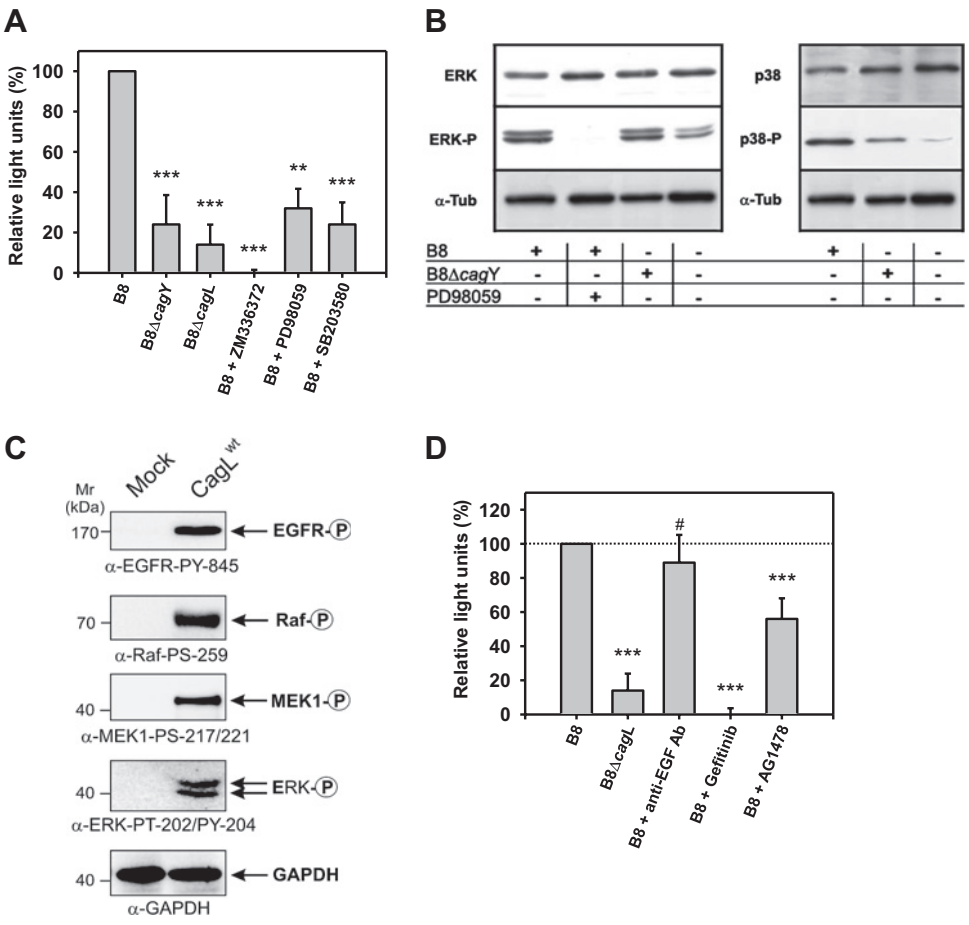

Figure 6 Epidermal growth factor receptor (EGFR)/Raf/mitogen activated protein kinase kinase (MEK)/extracellular signal regulated kinase (ERK) protein kinases are involved in *H pylori* induced gastrin promoter activation. (A) AM0 cells were preincubated with ZM336372 (50 μM), PD98059 (50 μM) or SB203580 (25 μM) and stimulated with *H pylori* B8 strain. (B) Western blot analyses were performed to show *H pylori* induced phosphorylation of Erk and p38. Erk signalling was inhibited by using PD98059 (100 μM). α-tubulin (Tub) served as a loading control. (C) Western blot analyses of AGS cells stimulated with recombinant *H pylori* wild-type (WT) CagL (5 μg) applying antibodies against phosphorylated forms of EGFR, Raf, MEK1 and ERK. α-GAPDH antibody was used as a loading control. (D) AM0 cells were preincubated with α-EGF antibody, gefitinib or AG1478 and stimulated with *H pylori* B8. Luciferase activities in relation to *H pylori* B8 WT (represents 100%) were plotted. The mean values ± SEM of at least three independent experiments are shown. Statistical analysis (Student's t test) was applied using SigmaStat software. ***p≤0.001; **p≤0.01; #p>0.05.

Our data demonstrate for the first time that *H pylori* uses a $\alpha v\beta_5$-integrin–ILK signalling complex to induce gastrin expression. We have shown that $\alpha v\beta_5$-integrin is a novel human receptor exploited by a bacterial pathogen to induce precancerous conditions in gastric epithelial cells. Kwok *et al* found that β_1-integrin functioned as an important receptor for binding of the *H pylori* T4SS to host cells. The authors demonstrated that this interaction triggers CagA translocation into the host cells and activates the downstream cascade via FAK and Src.[6] In the present study, we established that T4SS, but not β_1-integrin receptor or the FAK/Src cascade, plays a major role in gastrin promoter induction. In contrast, by means of silencing, blocking and immunoprecipitation experiments, a newly discovered interaction could be identified between *H pylori* WT and $\alpha v\beta_5$-integrin that was followed by an ILK dependent downstream signalling cascade. A series of experiments, including function blocking antibodies, siRNA and inhibitors conclusively revealed that *H pylori* induced gastrin promoter activation involves EGFR followed by an MEK/Erk signalling cascade, but seems independent of EGF itself.

Using a variety of mutant *H pylori* strains during infection, our data revealed that the major bacterial virulence factors CagA, VacA, BabA, SabA and AlpA/B are not required for induction of gastrin gene expression. However, binding of *H pylori* WT strains carrying an intact T4SS of the *cag*PAI was necessary to induce the gastrin promoter. This is in agreement with several patient studies demonstrating that the *H pylori* CagA status, linked to the presence of the entire *cag*PAI, is correlated with an increased plasma gastrin level in gastritis and gastric cancer patients.[58–61] Those observations raised the question which bacterial T4SS factor is essential for gastrin gene induction on binding to $\alpha v\beta_5$-integrin. Several lines of evidence presented here demonstrate that this factor is CagL. Firstly, deletion of *cag*L gene in *H pylori* causes loss of gastrin promoter activation during infection of AM0 cells. Secondly, immunoprecipitation experiments during infection demonstrate that CagL forms a physical complex with $\alpha v\beta_5$-integrin. Thirdly, purified recombinant CagL alone is sufficient to induce gastrin promoter activation on contact with AM0 cells. Fourthly, our Biacore protein–protein interaction studies in vitro demonstrate that CagL binds with very high affinity to $\alpha v\beta_5$ integrin (K_D=0.20 μM). Fifthly, recombinant CagL alone, on binding to $\alpha v\beta_5$-integrin, triggers profound activation of the EGFR→Raf→MEK→Erk signalling cascade upstream of the gastrin promoter.

Apart from the fact that CagL triggers $\alpha v\beta_5$-integrin mediated gastrin promoter activation, our study suggests that this signalling proceeds by an intriguing RGD independent mechanism. The first report demonstrating the importance of CagL, which is located at the surface of the T4SS injection pilus, inducing the translocation of the oncoprotein CagA in a RGD dependent manner,[6] let us originally propose that the CagL dependent activation of the gastrin promoter might be RGD dependent also. To our surprise, applying several different isogenic *H pylori* mutant strains that carry a mutated or total deleted RGD motif of the *cagL* gene, exhibited strong gastrin induction compared with WT levels. In line with these observations, both WT CagL and mutated $CagL^{RGA}$ bound with similarly strong affinity to $\alpha v\beta_5$-integrin, both during infection in vivo (as supported by IP experiments) and as

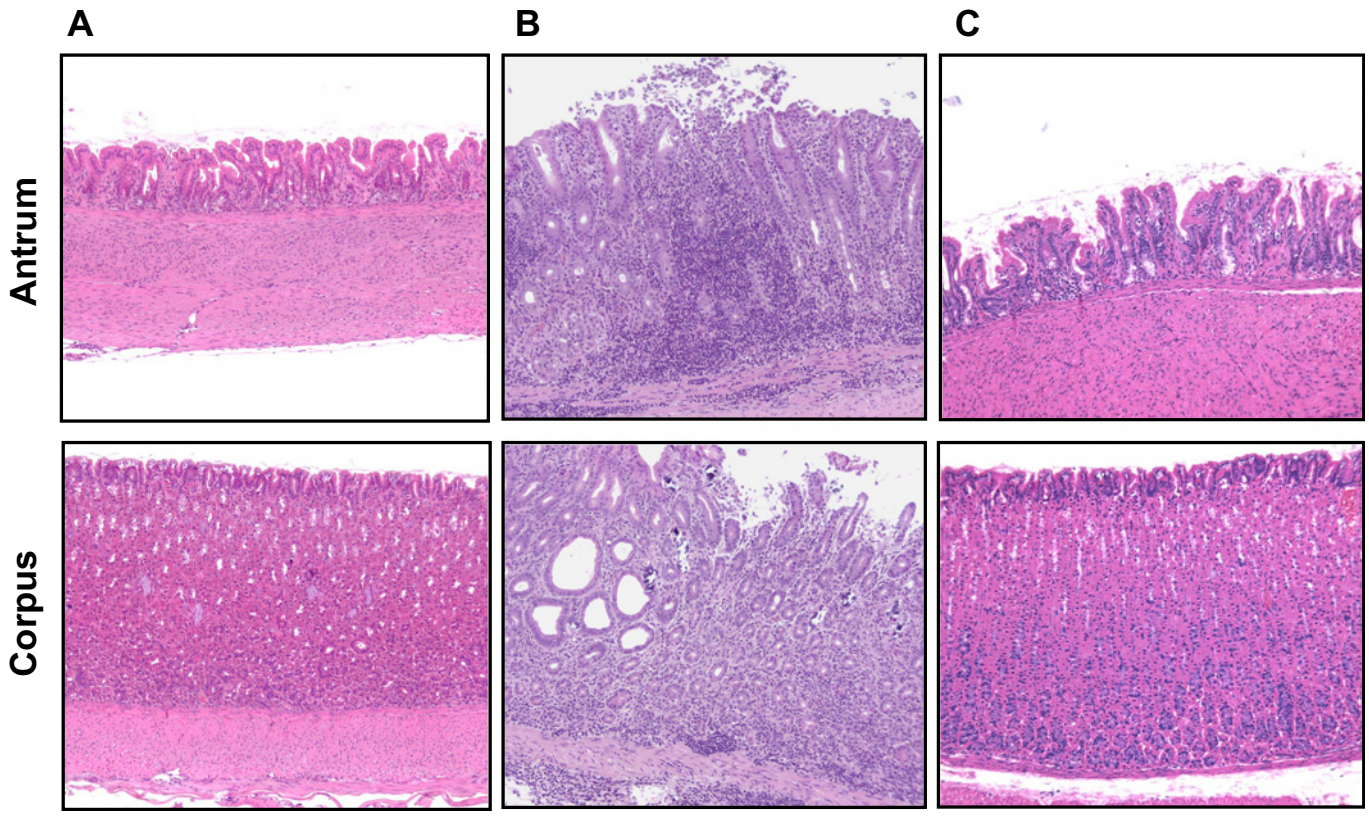

Figure 7 CagL deficiency prevents inflammation in corpus mucosa of infected Mongolian gerbils. Haematoxylin–eosin stained paraffin embedded antral and corpus tissue of (A) non-infected, (B) *H pylori* B8 and (C) isogenic B8Δ*cag*L mutant strain infected Mongolian gerbils for 8 weeks (original magnification ×10).

purified proteins in vitro (as supported by Biacore binding studies).

The development of gastric cancer is a highly complex process and proinflammatory cytokines play an important role in the progression of precancerous conditions. In our recently published animal study it was shown that *H pylori* B8 WT strain can induce an early and severe inflammation in antral and corpus mucosa that is associated with increased expression of proinflammatory cytokines.[32] In contrast, isogenic *H pylori* mutant strains with a defective T4SS revealed an early moderate to severe inflammation only in the antral but not in the corpus mucosa. This indicates that induction of early inflammation in the corpus mucosa by *H pylori* is part of a pathomechanism which causes later precancerous physiological changes, such as hypochlorhydria and hypergastrinaemia. These observations demonstrate that an infection of 8 weeks is sufficient to distinguish between bacterial factors harbouring a risk factor for developing cancerous conditions or not. As shown in the in vivo part of this study, Δ*cagL* mutants revealed strongly attenuated responses compared with WT bacteria. Thus CagL, a functional protein of the T4SS, is an essential bacterial ligand interacting with the host receptor family β-integrin to induce precancerous conditions.

The question arises how our newly discovered in vitro data can be extrapolated to understand the interactions of *H pylori* with G cells and gastrin production in vivo. It is known that *H pylori* is able to release proteases and other soluble factors that open the tight and adherence junctions of epithelial cells.[62] This enables the bacteria to reach the basolateral side of the cells where integrin is expressed. We assume a comparable mechanism regarding G cells and their stimulation by *H pylori*. This is not an early side effect of infection but requires a solid colonisation of *H pylori* with a severe inflammation associated with histopathological changes of the gastric mucosa. The occurrence of hypergastrinaemia in our in vivo experiments was based on a late physiological change due to an infection with *H pylori* type I strain.

Taken together, in this report we characterised a novel pathway leading to *H pylori* induced gastrin promoter activation and presented evidence for a new role of the CagL protein to induce the gastrin promoter in a $\alpha v\beta_5$-integrin dependent manner. These findings represent an important *H pylori* associated signalling cascade in gastrin gene induction. Using a combination of in vitro and in vivo experiments, we elucidated a *H pylori* induced upstream signalling pathway of the gastrin promoter via the EGFR→Raf→MEK→Erk cascade. In a time of increasing *H pylori* antibiotic resistance, the newly discovered interaction of *H pylori* CagL with $\alpha v\beta_5$-integrin could be a useful target in treating gastric precancerous conditions triggered by *H pylori* induced hypergastrinaemia.

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Competing interests None.

Contributors Conceived and designed the experiments: GR, SB; performed the experiments: TW, SHo, NT, SHu; analysed the data: GR, SB, TW, NS; contributed reagents/materials/analysis tools: SW, NS; wrote the paper: GR, SB.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1. **Suerbaum S,** Michetti P. Helicobacter pylori infection. *N Engl J Med* 2002;**347**:1175—86.
2. **Kuipers EJ,** Uyterlinde AM, Pena AS, *et al*. Long-term sequelae of Helicobacter pylori gastritis. *Lancet* 1995;**345**:1525—8.
3. **Backert S,** Meyer TF. Type IV secretion systems and their effectors in bacterial pathogenesis. *Curr Opin Microbiol* 2006;**9**:207—17.
4. **Covacci A,** Rappuoli R. Tyrosine-phosphorylated bacterial proteins: Trojan horses for the host cell. *J Exp Med* 2000;**191**:587—92.
5. **Hatakeyama M.** The role of Helicobacter pylori CagA in gastric carcinogenesis. *Int J Hematol* 2006;**84**:301—8.
6. **Kwok T,** Zabler D, Urman S, *et al*. Helicobacter exploits integrin for type IV secretion and kinase activation. *Nature* 2007;**449**:862—6.
7. **Poppe M,** Feller SM, Romer G, *et al*. Phosphorylation of Helicobacter pylori CagA by c-Abl leads to cell motility. *Oncogene* 2007;**26**:3462—72.
8. **Tammer I,** Brandt S, Hartig R, *et al*. Activation of Abl by Helicobacter pylori: a novel kinase for CagA and crucial mediator of host cell scattering. *Gastroenterology* 2007;**132**:1309—19.
9. **Wessler S,** Backert S. Molecular mechanisms of epithelial-barrier disruption by Helicobacter pylori. *Trends Microbiol* 2008;**16**:397—405.
10. **Tegtmeyer N,** Hartig R, Delahay RM, *et al*. A small fibronectin-mimicking protein from bacteria induces cell spreading and focal adhesion formation. *J Biol Chem* 2010;**285**:23515—26.
11. **Edkins JS.** The chemical mechanism of gastric secretion. *J Physiol* 1906;**34**:133—44.
12. **Schmitz F,** Goke MN, Otte JM, *et al*. Cellular expression of CCK-A and CCK-B/gastrin receptors in human gastric mucosa. *Regul Pept* 2001;**102**:101—10.
13. **Hakanson R,** Liedberg G. The role of endogenous gastrin in the activation of gastric histidine decarboxylase in the rat. Effect of antrectomy and vagal denervation. *Eur J Pharmacol* 1970;**12**:94—103.
14. **Chupreta S,** Du M, Todisco A, *et al*. EGF stimulates gastrin promoter through activation of Sp1 kinase activity. *Am J Physiol Cell Physiol* 2000;**278**:C697—708.
15. **Merchant JL,** Du M, Todisco A. Sp1 phosphorylation by Erk 2 stimulates DNA binding. *Biochem Biophys Res Commun* 1999;**254**:454—61.
16. **Subramaniam D,** Ramalingam S, May R, *et al*. Gastrin-mediated interleukin-8 and cyclooxygenase-2 gene expression: differential transcriptional and posttranscriptional mechanisms. *Gastroenterology* 2008;**134**:1070—82.
17. **Noble PJ,** Wilde G, White MR, *et al*. Stimulation of gastrin-CCKB receptor promotes migration of gastric AGS cells via multiple paracrine pathways. *Am J Physiol Gastrointest Liver Physiol* 2003;**284**:G75—84.
18. **Wroblewski LE,** Pritchard DM, Carter S, *et al*. Gastrin-stimulated gastric epithelial cell invasion: the role and mechanism of increased matrix metalloproteinase 9 expression. *Biochem J* 2002;**365**:873—9.
19. **Sun WH,** Zhu F, Chen GS, *et al*. Blockade of cholecystokinin-2 receptor and cyclooxygenase-2 synergistically induces cell apoptosis, and inhibits the proliferation of human gastric cancer cells in vitro. *Cancer Lett* 2008;**263**:302—11.
20. **Przemeck SM,** Varro A, Berry D, *et al*. Hypergastrinemia increases gastric epithelial susceptibility to apoptosis. *Regul Pept* 2008;**146**:147—56.
21. **Cui G,** Takaishi S, Ai W, *et al*. Gastrin-induced apoptosis contributes to carcinogenesis in the stomach. *Lab Invest* 2006;**86**:1037—51.
22. **Kidd M,** Tang LH, Modlin IM, *et al*. Gastrin-mediated alterations in gastric epithelial apoptosis and proliferation in a mastomys rodent model of gastric neoplasia. *Digestion* 2000;**62**:143—51.
23. **Willems G,** Vansteenkiste Y, Limbosch JM. Stimulating effect of gastrin on cell proliferation kinetics in canine fundic mucosa. *Gastroenterology* 1972;**62**:583—9.
24. **Wang TC,** Dangler CA, Chen D, *et al*. Synergistic interaction between hypergastrinemia and Helicobacter infection in a mouse model of gastric cancer. *Gastroenterology* 2000;**118**:36—47.
25. **Wang TC,** Koh TJ, Varro A, *et al*. Processing and proliferative effects of human progastrin in transgenic mice. *J Clin Invest* 1996;**98**:1918—29.
26. **Zavros Y,** Rieder G, Ferguson A, *et al*. Genetic or chemical hypochlorhydria is associated with inflammation that modulates parietal and G-cell populations in mice. *Gastroenterology* 2002;**122**:119—33.
27. **Koh TJ,** Goldenring JR, Ito S, *et al*. Gastrin deficiency results in altered gastric differentiation and decreased colonic proliferation in mice. *Gastroenterology* 1997;**113**:1015—25.
28. **Friis-Hansen L,** Sundler F, Li Y, *et al*. Impaired gastric acid secretion in gastrin-deficient mice. *Am J Physiol* 1998;**274**:G561—8.
29. **Sun FJ,** Kaur S, Ziemer D, *et al*. Decreased gastric bacterial killing and up-regulation of protective genes in small intestine in gastrin-deficient mouse. *Dig Dis Sci* 2003;**48**:976—85.
30. **Zhao CM,** Wang X, Friis-Hansen L, *et al*. Chronic Helicobacter pylori infection results in gastric hypoacidity and hypergastrinemia in wild-type mice but vagally induced hypersecretion in gastrin-deficient mice. *Regul Pept* 2003;**115**:161—70.
31. **Farnbacher M,** Jahns T, Willrodt D, *et al*. Sequencing, annotation, and comparative genome analysis of the gerbil-adapted Helicobacter pylori strain B8. *BMC Genomics* 2010;**11**:335.
32. **Wiedemann T,** Loell E, Mueller S, *et al*. Helicobacter pylori cag-Pathogenicity island-dependent early immunological response triggers later precancerous gastric changes in Mongolian gerbils. *PLoS One* 2009;**4**:e4754.
33. **Rieder G,** Merchant JL, Haas R. Helicobacter pylori cag-type IV secretion system facilitates corpus colonization to induce precancerous conditions in Mongolian gerbils. *Gastroenterology* 2005;**128**:1229—42.
34. **Tucker TP,** Gray BM, Eaton KA, *et al*. Helicobacter pylori induction of the gastrin promoter through GC-rich DNA elements. *Helicobacter* 2010;**15**:438—48.
35. **Backert S,** Schwarz T, Miehlke S, *et al*. Functional analysis of the cag pathogenicity island in Helicobacter pylori isolates from patients with gastritis, peptic ulcer, and gastric cancer. *Infect Immun* 2004;**72**:1043—56.
36. **Backert S,** Ziska E, Brinkmann V, *et al*. Translocation of the Helicobacter pylori CagA protein in gastric epithelial cells by a type IV secretion apparatus. *Cell Microbiol* 2000;**2**:155—64.
37. **Gebert B,** Fischer W, Weiss E, *et al*. Helicobacter pylori vacuolating cytotoxin inhibits T lymphocyte activation. *Science* 2003;**301**:1099—102.
38. **Odenbreit S,** Puls J, Sedlmaier B, *et al*. Translocation of Helicobacter pylori CagA into gastric epithelial cells by type IV secretion. *Science* 2000;**287**:1497—500.
39. **Sewald X,** Gebert-Vogl B, Prassl S, *et al*. Integrin subunit CD18 Is the T-lymphocyte receptor for the Helicobacter pylori vacuolating cytotoxin. *Cell Host Microbe* 2008;**3**:20—9.
40. **Sambrook J,** Russell D, eds. *Molecular cloning: a laboratory manual*. New York: Cold Spring Harbor Laboratory Press, 2001.
41. **Jimenez-Soto LF,** Kutter S, Sewald X, *et al*. Helicobacter pylori type IV secretion apparatus exploits beta1 integrin in a novel RGD-independent manner. *PLoS Pathog* 2009;**5**:e1000684.
42. **Ford MG,** Valle JD, Soroka CJ, *et al*. EGF receptor activation stimulates endogenous gastrin gene expression in canine G cells and human gastric cell cultures. *J Clin Invest* 1997;**99**:2762—71.
43. **Shiotani A,** Merchant JL. cAMP regulates gastrin gene expression. *Am J Physiol* 1995;**269**:G458—64.
44. **Higashi H,** Tsutsumi R, Muto S, *et al*. SHP-2 tyrosine phosphatase as an intracellular target of Helicobacter pylori CagA protein. *Science* 2002;**295**:683—6.
45. **Levi S,** Beardshall K, Swift I, *et al*. Antral Helicobacter pylori, hypergastrinaemia, and duodenal ulcers: effect of eradicating the organism. *BMJ* 1989;**299**:1504—5.
46. **Smith JT,** Garner A, Hampson SE, *et al*. Absence of a gastrin inhibitory factor in the IgG fraction of serum from patients with pernicious anaemia. *Gut* 1990;**31**:871—4.
47. **Godley JM,** Brand SJ. Regulation of the gastrin promoter by epidermal growth factor and neuropeptides. *Proc Natl Acad Sci U S A* 1989;**86**:3036—40.
48. **Odenbreit S,** Till M, Hofreuter D, *et al*. Genetic and functional characterization of the alpAB gene locus essential for the adhesion of Helicobacter pylori to human gastric tissue. *Mol Microbiol* 1999;**31**:1537—48.
49. **Mahdavi J,** Sonden B, Hurtig M, *et al*. Helicobacter pylori SabA adhesin in persistent infection and chronic inflammation. *Science* 2002;**297**:573—8.
50. **Linden S,** Nordman H, Hedenbro J, *et al*. Strain- and blood group-dependent binding of Helicobacter pylori to human gastric MUC5AC glycoforms. *Gastroenterology* 2002;**123**:1923—30.
51. **Bagnoli F,** Buti L, Tompkins L, *et al*. Helicobacter pylori CagA induces a transition from polarized to invasive phenotypes in MDCK cells. *Proc Natl Acad Sci U S A* 2005;**102**:16339—44.
52. **Cover TL,** Blaser MJ. Purification and characterization of the vacuolating toxin from Helicobacter pylori. *J Biol Chem* 1992;**267**:10570—5.
53. **Rohde M,** Puls J, Buhrdorf R, *et al*. A novel sheathed surface organelle of the Helicobacter pylori cag type IV secretion system. *Mol Microbiol* 2003;**49**:219—34.
54. **Harburger DS,** Calderwood DA. Integrin signalling at a glance. *J Cell Sci* 2009;**122**:159—63.
55. **Hocker M.** Molecular mechanisms of gastrin-dependent gene regulation. *Ann N Y Acad Sci* 2004;**1014**:97—109.
56. **Dockray GJ.** Topical review. Gastrin and gastric epithelial physiology. *J Physiol* 1999;**518**:315—24.
57. **Fox JG,** Wang TC, Rogers AB, *et al*. Host and microbial constituents influence Helicobacter pylori-induced cancer in a murine model of hypergastrinemia. *Gastroenterology* 2003;**124**:1879—90.
58. **Konturek PC,** Hartwich A, Zuchowicz M, *et al*. Helicobacter pylori, gastrin and cyclooxygenases in gastric cancer. *J Physiol Pharmacol* 2000;**51**:737—49.
59. **Kim JH,** Park HJ, Cho JS, *et al*. Relationship of CagA to serum gastrin concentrations and antral G, D cell densities in Helicobacter pylori infection. *Yonsei Med J* 1999;**40**:301—6.
60. **Konturek SJ,** Starzynska T, Konturek PC, *et al*. Helicobacter pylori and CagA status, serum gastrin, interleukin-8 and gastric acid secretion in gastric cancer. *Scand J Gastroenterol* 2002;**37**:891—8.
61. **Saruc M,** Demir MA, Kucukmetin N, *et al*. Histological and clinical predictive value of determination of tissue CagA status by PCR in Helicobacter pylori infected patients; results of the large population based study in western Turkey. *Hepatogastroenterology* 2002;**49**:878—81.
62. **Hoy B,** Lower M, Weydig C, *et al*. Helicobacter pylori HtrA is a new secreted virulence factor that cleaves E-cadherin to disrupt intercellular adhesion. *EMBO Rep* 2010;**11**:798—804.

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