Role of C-Raf/ MEK1/ ERK2 in *Helicobacter pylori*-induced gastrin promoter activity

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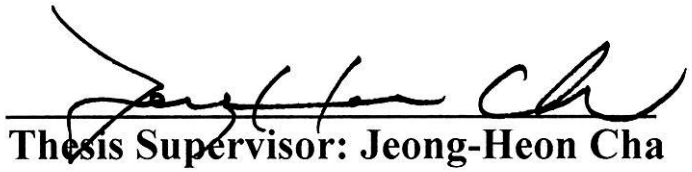
This certifies that the Doctoral dissertation of Niluka Darshani Gunawardhana is approved.



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Niluka Darshani Gunawardhana

CONTENTS

III. RESULTS

List of Figures

List of Tables

ABSTRACT

Role of C-Raf/ MEK1/ ERK2 in *Helicobacter pylori*-induced gastrin promoter activity

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Introduction: *Helicobacter pylori* is a gram negative bacterium, specialized in the colonization of human stomach and a major risk factor for development of gastric cancers. It has been observed in *H. pylori*-infected patients that gastrin secretion stimulated by *H. pylori* leads to hypergastrinemia and as consequences, chronic gastritis and gastric atrophy. Gastrin is an important

hormone in human stomach which mainly regulates gastric acid secretion. In addition, it is also involved in growth and differentiation of gastric epithelial cells. *H. pylori*-induced hypergastrinemia is known as a major risk factor for the development of gastric cancers. Moreover, it has been described that *H. pylori* modulates mitogen activated protein (MAP) kinase pathway, one of the major epithelial cell signaling pathways in humans, which regulates gastrin expression. However, the molecular mechanism, especially how each isotypes of mitogen activated protein kinase pathway are involved in *H. pylori*-induced gastrin expression, is not fully understood.

Purpose: The aim of this study is to explore the molecular mechanism underlying the *H. pylori*-induced gastrin expression.

Materials and methods: The expression level of human gastrin promoter was measured using G240-Luc cells which were constructed by the stable transfection of gastrin promoter-luciferase reporter into AGS cells. In order to identify *H. pylori* components responsible for the induction of gastrin expression, isogenic mutants of *H. pylori* G27 wild type; G27Δ*cagA*, G27Δ*cagL* and G27ΔPAI were constructed. G27Δ*cagA* strain lacks *cagA* gene which encodes the *H .pylori* major virulence factor CagA, while G27Δ*cagL* strain lacks *cagL* gene which is essential for the *H. pylori* type IV secretion

system. G27ΔPAI strain has an entire deletion of *cag* pathogenicity island encodes the type IV secretion system. After G240-Luc cells were infected by *H. pylori* G27 or isogenic mutant strains, luciferase activities expressed by the cells were measured and compared. In addition, G240-Luc cells were stimulated with growth factors; Heparin-binding epidermal growth factor like-growth factor (HB-EGF) and Epidermal growth factor (EGF). To knockdown the expression of each signal molecule of MAP kinase pathway, specific siRNAs targeting A-Raf, B-Raf, C-Raf, MEK1, MEK2, ERK1 and ERK2 were used. To verify protein expression knockdown by siRNA treatment, western blot was performed.

Results and discussion: G240-Luc cells infected with *H. pylori* G27 and G27Δ*cagA* strains showed increased luciferase expression levels and hence increased gastrin promoter activity. In contrast to that, G27Δ*cagL* and G27ΔPAI *H. pylori* strains were unable to stimulate the gastrin promoter activity, suggesting that *H. pylori* type IV secretion system apparatus is important in gastrin promoter activation. In addition, HB-EGF treatment showed a significant gastrin promoter induction which was in similar level to the gastrin promoter induction by EGF treatment, the known agonist of gastrin expression. Both *H. pylori*-stimulated as well as HB-EGF-stimulated gastrin promoter activities were reduced in the presence of EGFR kinase inhibitor,

AG1478. This result suggests that EGFR activation is important in both *H. pylori* and HB-EGF-induced gastrin promoter activity. As it has been reported that HB-EGF expression was induced by *H. pylori*, this result leads to speculate that the activation of EGFR via HB-EGF may play a role in *H. pylori*-induced gastrin expression. C-Raf siRNA treatment showed a significant reduction in *H. pylori*-stimulated gastrin promoter activity, while A-Raf and B-Raf siRNA treatments didn't show such reduction in the gastrin promoter activity. This suggests that signal transduction, which lead to gastrin promoter activation by *H. pylori* is mediated via C-Raf. In addition to that, MEK1-knockdown cells also showed a significant reduction in promoter activity, but knockdown of MEK2 didn't show any reduction in gastrin promoter activity. This result implies that MEK1 is important in *H. pylori*-stimulated gastrin expression. This further implies that, MEK1 and MEK2 may play different roles in *H. pylor*i-induced gastrin expression, even though MEK1 and MEK2 proteins function redundantly in general. In the context of ERK, knockdown of ERK2 exhibited significant reduction in gastrin promoter activity, suggesting that ERK2 plays a major role in *H. pylori*-stimulated gastrin promoter activation. Surprisingly, the knockdown of ERK1 showed a significant induction in *H. pylori*-induced gastrin promoter activity. This result leads to speculate that ERK1 may exert an inhibitory effect on *H. pylori*-stimulated gastrin promoter activity, possibly by forming heterodimers with

ERK2 and ERK2 homodimers may act as potent gastrin promoter activators.

Conclusion: *H. pylori* modulates ERK signal transduction pathway to stimulate gastrin expression via EGFR, C-Raf, MEK1 and ERK2. Moreover, this study reveals for the first time that HB-EGF can stimulate the gastrin promoter activity and thus, EGFR activation via HB-EGF may also play a role in *H. pylori*-induced gastrin expression. Intriguingly, ERK1 and ERK2 act differently in *H. pylori*-stimulated gastrin expression. Furthermore, the knowledge of the inhibitory effect of ERK1 in gastrin promoter activation will be helpful to lessen the effect of *H. pylori*-stimulated gastrin expression. This current understanding of the involvement of MAPK pathway would facilitate the development of therapeutic strategies to reduce *H. pylori*-induced hypergastrinemia as well as the gastric cancer development.

Key words: *Helicobacter pylori*, Gastrin, MAPK pathway, Raf, MEK, ERK1, ERK2

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I. INTRODUCTION

Gram negative, spiral shaped, microaerophilic bacterium *Helicobacter pylori* is the dominant species of human gastric niche. Genetic studies reveal that, this bacterium has accompanied with human for at least 58,000 years, suggesting the historical relationship with humans [1]. Even though, approximately half of the world's population infected by this bacterium, most

individuals remains asymptomatic [2]. *H. pylori* is the major causative agents of chronic gastritis, peptic ulcers, gastric adenocarcinoma and mucosa associated lymphoid tissue (MALT) lymphoma [3,4]. As *H. pylori* infection is associated with the increased risk of developing gastric cancers, which is known as the second leading cause of cancer related deaths worldwide, in 1994 the international research agency on cancer (IRAC) has classified *H. pylori* as a 'type 1 carcinogen'[5].

H. pylori possesses several virulence factors which are important for the pathogenesis [6]. The *cag* pathogenicity island (*cag*PAI) which consists of a group of about 32 genes is a major virulence factor of *H. pylori*. This encodes for Type IV secretion system (T4SS) that assists the injection of the only known effector protein CagA into host cells [7]. The translocated CagA is tyrosine phosphorylated at the C-terminus repeat sequence by src/Ab1 kinases [8,9] and has been reported to regulates various cellular responses, including actin-cytoskeletal rearrangements, cell elongation, scattering, motility and pro-inflammatory cytokine activation [10-12]. Various other structural proteins are important for the formation of T4SS pilus. The CagL, a VirB5 orthologue, is one such protein. It has been found to locate at the tip of the T4SS pilus and interact with the integrin α5β1 receptors of gastric epithelial cells [13]. CagL binding to integrin α5β1was found to be required for both pilus extension and CagA translocation. VacA, is another extensively studied *H. pylori* virulence

factor which induces vacuolations in the cells and also involved in disruption of mitochondrial functions, stimulation of apoptosis and blockade of T-cell proliferation [14]. *H. pylori* outer membrane proteins such as OipA, BabA, Alp A/B and SabA have been reported to play major roles in bacterial adhesion and diseases causation [15-17].

Infection with *H. pylori* is known to induce serum hypergastrenemia other than its known major clinical outcomes [18]. Gastrin is an important polypeptide hormone, which is mainly secreted by the G cells in the antro-pyloric mucosa. Its main function is to stimulate gastric acid secretion by the parietal cells of the stomach during the food digestion, thereby maintain the appropriate acidic pH for the proper function of gastric digestive enzymes. Gastrin is translated as a 101 amino acid precursor and undergoes sequential processing to form the amidated end product: gastrin 17 and gastrin 34. After secreted from G cells, it transported to its site of action, oxyntic mucosa, in an endocrine manner. There it binds to cholecystokinin-2 receptors on enterochromaffin-like cells (ECL) to stimulate histamine release, which then interacts with the parietal cell to induce gastric acid secretion.

Factors influencing the gastrin secretion

The main stimulus for gastrin secretion is presence of dietary proteins and amino acids in the stomach. In addition to that, gastrin-releasing peptide/ bombesin stimulates gastrin protein release in response to vagal nerve

stimulation [19]. Also beta-adrenergic agents, cholinergic agents and hypercalcemia are other stimulants for gastrin secretion. When the stomach acidity is increased (pH below 3), delta (D) cells in the antrum, secrete somatostatin which then inhibits gastric acid secretion. Also gastric inhibitory peptide (GIP), secretin and calcitonin can negatively regulate gastrin secretion. In addition to that, physiological stimuli also can regulate expression of the gastrin gene. Hence several studies have been focused on the transcriptional regulation of the human gastrin gene. Early studies identified epidermal growth factor (EGF) as a potent regulator of gastrin expression [20]. A GC-rich gastrin EGF response element (gERE) located at -68 bp was identified as the region mediates the responsiveness of the gastrin gene to EGF receptor ligands [21]. In addition to that, binding sites of several other regulatory elements have been identified in the gastrin promoter [22-24].

Other than the stimulation of gastric acid secretion by parietal cells, it has been shown that gastrin is involved in various vital cellular processes such as proliferation, apoptosis, angiogenesis, motility and increased invasion [25,26].

Gastrin is well known for its growth promoting effect in both normal and malignant gastrointestinal tissues as well as gastric epithelial cells [27]. Several studies have proved the gastrin's growth promoting effect *in vitro* and *in vivo* [27-29]. In early studies, patients with high levels of circulating gastrin showed significant hyperplasia of the gastric mucosa [30]. In addition to that gastrin

deficient mice showed significantly reduced levels of basal and stimulated acid, hence atrophy of gastric mucosa [31], while INS-GAS mice which produce excess gastrin peptide, showed increased proliferation of gastric mucosa [32].

Gastrin increases the proliferation of gastric cells through transcriptional activation of a series of genes. Among those genes, Regenerating islet-derived 1 alpha (REG1α) is one, which has identified in gerbil model experiments [33]. Not only that, it has been reported that gastrin can induces HB-EGF, and amphiregulin genes and those growth factors play a role in trophic action of gastrin [34].

Gastrin in diseases

Hypergastrinemia is the presence of serum gastrin levels above the normal level (>150 pg/ml). This can occur secondary to uncontrolled gastrin production or in response to decreased acid secretion. Uncontrolled excess gastrin secretion can be seen in gastrin secreting tumours or gastrinomas. They arise sporadically or as part of the multiple endocrine neoplasia-type 1(MEN-1) syndrome. Most of the tumours are malignant and the fasting serum gastrin level in these patients is about 1000 pg/ml [35]. Zollinger-Ellison syndrome is a clinical condition characterized by acid hyper-secretion due to a gastrinoma [36]. Hypergastrinemia in response to decreased acid secretion typically occurs in patients who take proton-pump inhibitors or with chronic atrophic gastritis. Proton pump inhibitors (PPI) are potent inhibitors of gastric acid secretion,

which block the parietal cell's apical proton pump, the final pathway for acid secretion. As a consequence, chronic PPI users develop modest degrees of hypergastrinemia (200–400 pg/ml) [37].

Chronic atrophic gastritis is a process of chronic inflammation of the gastric mucosa, leading to loss of gastric glandular cells and their eventual replacement by intestinal and fibrous tissues. *H. pylori* infection is a major contributor for chronic atrophic gastritis [38,39].

***H. pylori* and gastrin**

H. pylori is an important risk factor for gastric carcinogenesis and gastric atrophy. It has been observed that *H. pylori* infection can cause hypergastrinemia and decreased acid secretion [40]. Also it has been demonstrated that, a reduction in fasting serum gastrin concentration after eradication of *H. pylori* [41]. Several *in vivo* experiments using rodent models also have proved the relationship between hypergastrinemia associated pathology and *H. pylori.* INS-GAS mice are transgenic mice that over express gastrin gene and show elevated level of serum amidated gastrin [42]. Uninfected INS-GAS mice developed gastric malignancies for about 2 years (18-20 months), but this process has been accelerated with *Helicobacter felis* or *Helicobacter pylori* infection resulting gastric cancers within 6 months [43,44]. In contrast to that, the outcomes of gastrin deficiency have been explored using gastrin knockout mice. These mice are characterised by reduced parietal

cell numbers and are predisposed to bacterial colonization of the stomach [45]. When these mice were infected with *cagA*$^+$ and *vacA*$^+$ *H. pylori* there was an alteration in acid secretion, thought to be stimulated by a vagal response, but no increased risk of tumour development is observed after 6 months [46].

Furthermore, several attempts have been carried out to delineate the mechanism underlying the hypergastrinemia caused by *H. pylori* infection. It has been reported that direct contact of live *H. pylori* with human cells is sufficient to induce the gastrin gene expression [47]. Also it has been noted that, during *H. pylori* induced inflammation, cytokines such as IL-8, TNFα and IL1β have been implicated in increasing gastrin production from G cells [48,49].

Mitogen-activated protein kinase (MAPK) pathways

One group of important signaling molecules activated by *H. pylori* is mitogen-activated protein kinases (MAPK). Mitogen-activated protein kinase (MAPK) pathways are key signaling pathways involved in the regulation of fundamental cellular processes such as growth, proliferation, differentiation, migration and apoptosis [50]. So far the most extensively studied groups of mammalian MAPKs are the ERK1/2, JNKs, and p38 isoforms.

Each group of conventional MAPKs is composed of a set of three evolutionarily conserved, sequentially acting kinases: a MAPKK kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK. The activated MAP kinases phosphorylate numerous substrates including transcriptional factors,

protein kinases, phosphatases and other functional proteins.

Out of the MAPK pathways, Raf-MEK-ERK pathway, which is the best studied MAPK pathway, regulates a diverse array of cellular programs such as proliferation, differentiation, apoptosis, and transformation to the cancerous state [51].

This pathway is generally regulate through the activation of cell surface receptors which are called "Receptor Tyrosine Kinases" (RTK). There are four types of receptors known as ErbB1 (EGFR), ErbB2 (Her2), ErbB3 (Her3), ErbB4 (Her4). Also there are seven known ligands for EGFR as follows; Epidermal growth factor (EGF), Heparin-Binding EGF-like growth factor (HB-EGF), Transforming growth factor alpha (TGF-α), Epiregulin, Amphiregulin, Betacellulin and Epigen [52].

Once RTK has been activated by ligand binding, series of adapter protein activation leads to activate the inactive Ras into GTP bound active Ras. Activated Ras phosphorylates its downstream substrate Raf. Raf kinases are a family of serine/threonine kinases and in humans there are three members known as A-Raf, B-Raf and C-Raf (Raf-1) [51]. Raf proteins have restricted substrate specificity and phosphorylate MEK1 and MEK2 which are known as dual specificity protein kinases. Raf-mediated phosphorylation of MEK1 and MEK2 occurs on two neighboring serine residues in the activation segments; Ser218, Ser222 in MEK1 and Ser222, Ser226 in MEK2 [53]. Upon activation,

MEK1 and MEK2 phosphorylate their well known substrates, extracellular signal regulated kinase (ERK) 1 and 2, on specific tyrosine and threonine residues. Human ERK1 and ERK2 have about 84% sequence identity and share many cellular functions [54]. Activated ERKs can phosphorylate many cytoplasmic targets as well as nuclear targets such as kinases, phosphatases, transcription factors and cytoskeletal proteins.

It is widely reported that the involvement of MAPK signal pathways in *H. pylori*-mediated cellular functions such as proliferation, transformation and apoptosis [55-57] . In addition to that, MAPK pathway is important in *H. pylori*-induced IL-8 expression as well as c-*fos* and *c-jun* proto-oncogene expression [58,59]. In relation to gastrin expression, experiments which have been carried out using kinase inhibitors of MAPK pathway revealed that MAPK pathway is important in gastrin promoter induction by *H. pylori* as well as with EGF [60-62].

There are some limitations of the usage of kinase inhibitors. Lack of specificity is a main limitation. In addition to that, those inhibitors may affect proteins of similar confirmation [63]. In the situations where kinase inhibitors are used to inhibit signal molecules, it's difficult to inhibit specific isoforms of the molecules. But usage of siRNA has shown a promising method to overcome those limitations.

Small interfering RNA (siRNA) is a class of double-stranded RNA molecules, and about 20-25 base pairs in length. It interferes with the expression of

specific genes with complementary nucleotide sequences in RNA interference (RNAi) pathway. siRNA fulfils the gene silencing by breaking down mRNA hence no translation occurs.

As the importance and the involvement of each isoform of ERK signal cascade in *H. pylori*-induced gastrin promoter induction has not been identified clearly and specifically, this study was carried out to explore the molecular mechanism underlying this promoter activation.

III. MATERIAL AND METHODS

1. Construction of human gastrin promoter expressing the luciferase reporter

Segments from -240 bp of gastrin promoter with the first exon [22] (Figure 1) was amplified with a proof reading *pfu* polymerase (iNtRon) using human genomic DNA as template. The 3' PCR primer contained an XhoI site and was +60 5'-CCC CTC GAG CTG CAG AGC TGG GAG GTG TG-3'. The 5' PCR primer contained SacI site and was as -240 5'-AAA GAG CTC AGC TGG AGA GCT GCC GCC-3' (Product size-303 bp).

Another construct which contains 1.3 kb human gastrin promoter expressing luciferase reporter was constructed as follows. The 1.3 kb gastrin promoter sequence was amplified by *pfu* polymerase using human genomic DNA as template. The 3' PCR primer and 5' primer contained XhoI and SacI enzyme sites respectively. The 3' primer was 5'-CCC CTC GAG CTG CAG AGC TGG GAG GTG TG-3' and the 5' primer was 5'-GGG GAG CTC CAA TGA CGC ATG AAC AGG-3' (Product size-1374 bp). Amplified gastrin promoter fragments were ligated upstream of pGL3 basic vector (Promega). Constructs were confirmed as correct by sequence analysis.

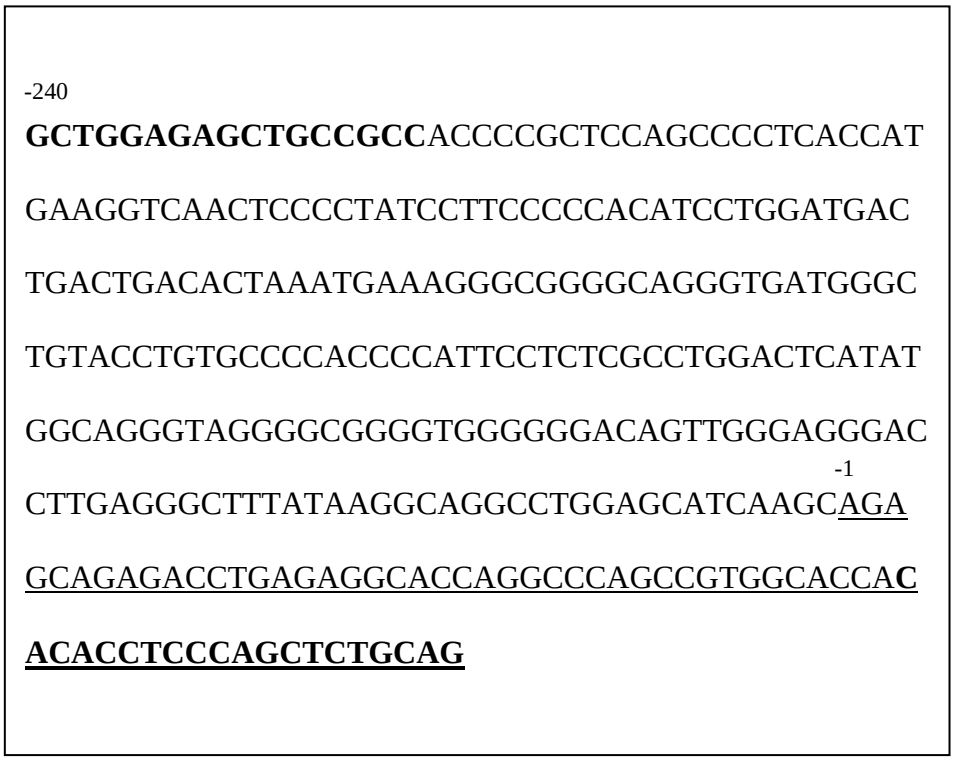


Figure 1. Sequence of human gastrin promoter with the first exon.

The sequence of the gastrin promoter from -240 bp to +60 bp from the transcription initiation site has shown. Primers used in this study are indicated by boldfaced characters. Underlined sequence is corresponding to the sequence of first exon.

2. Making stable AGS cells (G240-Luc cells)

AGS (Human Gastric adenocarcinoma, ATCC CRL 1739) cells were cultured in Roswell Park Memorial Institute medium (RPMI-1640, Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Gibco), 1% penicillin/streptomycin (Gibco, 15140-122) at 37 ℃ in a humidified incubator (Thermo Electron Corporation) containing 5% CO_2.

Gastrin promoter expressing luciferase reporter constructs (240 bp or 1.3 kb) were co-transfected into AGS cells in a 3:1 ratio with the pcDNA3 plasmid and transfected cells were selected with 400 μg/ml of G418 (Sigma-A 1720). Cells which expressed higher luciferase activities were selected and pooled. AGS cells stably transfected with a plasmid containing 240 bp of the human gastrin promoter-expressing the luciferase reporter were named as "G240-Luc" cells, while AGS cells stably transfected with a plasmid containing 1.3 kb of the human gastrin promoter-expressing the luciferase reporter were named as "G1.3-Luc" cells. When perform the experiments, G240-Luc cells were maintained in 300 μg/ml of G418 and subsequently treated with siRNA, stimulated with growth factors or infected with *H. pylori*.

3. *H. pylori* strains and culture

H. pylori strain G27 wild type (WT) and its isogenic mutants G27Δ*cagA*, G27Δ*cagL*, G27ΔPAI were used in this study. In addition to that *H. pylori*

strains 26695, PMSS1 and as a clinical *H. pylori* strain K74 were used. K74 *H. pylori* strain (*cagA*+, *vacA*+) has isolated from a female Korean patient diagnosed as gastritis.

All *H. pylori* strains were cultured on antibiotic supplemented horse blood agar plates in microaerophilic atmosphere. Microaerophilic condition was generated by using a CampyGen sachet (Oxoid, Wesel, Germany) in a gas pack jar. For liquid cultures, *H. pylori* was grown in Brucella broth (Becton, Dickinson and Company) containing 10% FBS (Gibco) and 10 μg/ml Vancomycin (Sigma) with shaking in 110 rpm in a microaerophilic condition at 37 ℃.

4. *H. pylori* isogenic mutant construction

G27Δ*cagA* mutant was generated as described previously [64]. In brief p729, a plasmid containing chloramphenicol resistance cassette in the middle of *cagA* was transformed in to G27 WT *H. pylori* by natural transformation. G27ΔPAI mutant was also constructed as described elsewhere [65]. Briefly, p730 construct which has a replacement of PAI gene with kanamycin resistance gene (*aphA-3* from *Camphylobacter coli*) was introduced in to *H. pylori* G27 naturally. Transformed clones were selected using serum plates supplemented with chloramphenicol 25 μg/ml and kanamycin 25 μg/ml, respectively.

Mutants were confirmed by both sequencing and PCR analysis. G27Δ*cagA*

PCR analysis was performed using cagA-F-(13): 5’-GTA AGG AGA AAC AAT GAC TAA CG-3’ and cag seq 6: 5’-GCT TCA GCT ACA GCT TTA TTG A-3’, while G27ΔPAI mutants were confirmed using Del-PAI-F: 5’-CCA AAT TTT ATA GGA TTC GCG CTC-3’ and GRace1: 5’-GGT TGC ACG CAT TTT CCC-3’ (corresponding fragment sizes were ~ 3 kb each).

G27Δ*cagL* and K74Δ*cagL* mutants were constructed as follows. Flanking areas of G27 and K74 *H. pylori cagL* were amplified using *pfu* polymerase PCR. PCR products were generated using G27 *H. pylori* genomic DNA as template and using the primers for 5’-*cagL* fragment: cagL outside F(500) : 5’-AAG TGG CTA TGC AAA AAG CGA CCC-3’ and cagL XhoI SmaI F(2): 5’-TGT CAA CTC GAG CCT CCC GGG ATG ATT TTT CTG AGA CGA CAA G-3’ and 3’-cagL fragment: cagL XhoI SmaI R(2): 5’-AAT CAT CCC GGG AGG CTC GAG TTG ACA ATA ACT TTA GAG CTA GC-3’ and cagL outside R(500) : 5’-GTG CCT GAT GAG TGG AGA ACG CC-3’. To amplify flanking areas of K74 following primers were used. To obtain 5’-*cagL* fragment, cagL outside F(500)1 5’-AAG TGG CTA TGC AAA AAG CGA CCC-3’ and cagL XhoI-SmaI R-K74 5’-TTG TTG TCT CAG AAA AAT TAT CCC GGG AGG CTC GAG TTG ACA ATA ACT TTA GAG CTAG-3’ were used. To obtain 3’-cagL fragment, cagL XhoI-SmaI F-K74 5’-TAG CTC TAA AGT TAT TGT CAA CTC GAG CCT CCC GGG ATA ATT TTT CTG AGA CAA CAA G-3’ and cagL outside R(500)1-7.13 5’-GAG TGG AGA

ACA CCT GAA ATT G-3' were used. XhoI and SmaI restriction enzyme sites were incorporated into the primers of 5'-*cagL* fragment and the 3'-*cagL* fragment in order to delete the c*agL* by Splicing by Overlap Extension (SOEing) PCR. Fused 5' and 3'-*cagL* fragments were cloned into pGEM-T Easy vector (Promega). To insert antibiotic selection marker, *kan-sacB* cassette was obtained by XhoI and SmaI restriction enzymes digestion of pKSF-II plasmid [66]. The *kan-sacB* and *cagL* flanking fragments were ligated using T4 ligase (Promega). The correct nature of the plasmid was verified by enzyme digestion. To create G27Δ*cagL H. pylori*, pG27Δ*cagL*:kan-sacB construct was introduced into *H. pylori* G27 through natural transformation, while to create K74Δ*cagL H. pylori*, K74 WT *H. pylori* were transformed with pK74Δ*cagL*:kan-sacB plasmid naturally. The transformed bacteria were selected on serum plates, supplemented with 25 µg/ml kanamycin and further screened on serum plates supplemented with 5% sucrose. Single colonies of kanamycin-resistant, sucrose-sensitive *H. pylori* were verified by PCR amplification and sequencing of the genomic locus.

5. *In vitro* Gastrin promoter stimulation

G240-Luc cells, (1 x 10^5) were seeded in 12 well cell culture plates (Nunc) with RPMI medium supplemented with 10% FBS only. When the cells were at 70% confluence, they were maintained in serum-free and antibiotic-free RPMI

media for the duration of bacterial co-culture experiment. Two hours prior to infection media was changed again into serum and antibiotic free RPMI media. Bacterial suspensions of WT and mutant *H. pylori* strains at an MOI of 100:1 were used to stimulate the cells. In experiments with the growth factors, Gas-Luc cells were incubated in a serum free RPMI medium for 24 hrs prior to the growth factor treatment and human recombinant EGF or HB-EGF (R&D Systems) was treated onto the cells at 10 nM concentration to stimulate the gastrin promoter. All experiments were carried out in 3-4 individual experiments, in triplicates.

6. AG1478 treatment

After 24 hrs serum starvation, G240-Luc cells were pre-treated with 1 μM AG1478 (Calbiochem). Control cells were treated with Dimethyl Sulfoxide (DMSO, Amresco, USA) in the same volume of AG1478. After 30 minutes of inhibitor treatment, cells were infected with *H. pylori* or treated with HB-EGF.

7. siRNA sequences and transfection

G240-Luc cells were transfected with specifically designed small interfering RNAs (siRNA) to knockdown A-Raf, B-Raf [67], C-Raf, MEK1, MEK2 [68], ERK1 and ERK2 [69]. Table 1 shows the siRNA sequences used in this study. Low passage G240-Luc cells were ($1x\ 10^5$) were plated on 12 well plates in

1 ml RPMI growth media supplemented with 10% FBS the night before transfection. Transfection was carried out using 40 pmol/ml siRNA with Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's instructions. Six hours post-transfection the media was changed to growth media with 10% FBS. After 48 hrs of transfection, cells were infected with *H. pylori* for 5 hrs.

Table 1. siRNA sequences used in this study

Gene	Sense strand sequence	Reference
A-Raf	5'-CGAGAUCUCAAGUCUAACAtt -3' 5'-GCUUCCAGUCAGACGUCUAtt -3' 5'-GGACUCCUCUCUUUCUUCAtt -3'	Santa Cruz sc-29615
B-Raf	5'-AAAGAATTGGATCTGGATCAT-3'	65
C-Raf	5'-UAGUUCAGCAGUUUGGCUAtt -3'	Santa Cruz sc-29462
MEK1	5'-GCT TCTATGGTG CGTTCTACA -3' 5'-GATTACATAGTCAACGAGCCT-3'	66
MEK2	5'-TGGACTATATTGTGAACGAGC-3' 5'- CCAACATCCTCGTGAACTCTA-3'	66
ERK1	5'-AACUUGUACAGG UCAGUCU -3' 5'-AGAGACUGUAGGUAGUUUCUU-3'	67
ERK2	5'- AAUAAGUCCAGAGCUUUGG -3' 5'- AGCUUGUAAAGAUCUGUUUUU-3'	67
Non-targeting	5'- AAACCGUCGAUUUCACCCGGG -3'	66

8. Luciferase assay

Five hours after stimulation, carefully removed the growth medium from cells to be assayed. Cells were rinsed with 1xphosphate buffered saline (PBS) and removed as much of the PBS rinse as possible. Cells were lysed with 1xpassive lysis buffer (Promega Cat. # E1941). For each well 120 µl of 1xpassive lysis buffer was added and culture plates were rocked several times to ensure complete coverage of the cells with lysis buffer. Cells were incubated with lysis buffer for 10 minutes on ice. Then, the cells were scraped from the culture plates and transfer into eppendorf tubes and kept on ice. After that, the tubes with cell lysates were vortexed for about 10 seconds, and centrifuged at 12,000xg for 2 minutes at 4 ℃. The supernatants were transferred into new tubes subsequently dispense 10 µl cell lysate into micro-well pate (SPL life sciences). In order to measure the luciferase expression, 50 µl of Luciferase assay reagent (Promega) was added on to the cell lysates in the micro-well plate and mixed by tapping onto the plate briefly. The luminometer was programmed to perform 2 second measurement delay and 10 second measurement read and luciferase activity was measured. Luciferase activities were measured using a Promega luciferase assay system (Cat.# E1501) and a Centro XS^3 microplate luminometer LB 960 (Berthold Technologies). For normalization, total protein concentrations in cell lysates were determined by bicinchoninic acid assay (Pierce Biochemicals).

9. Western blot analysis

Cells were washed with 1x phosphate buffered saline (PBS) and then lysed as described by the manufacturer. Ten to twenty microgram samples of cell lysate was mixed with sample buffer; boiled for 5 minutes and then loaded on 10% SDS-polyachralamyde gel. The separated proteins were transferred onto a polyvinylidene difluoride (PVDF- Millipore) membrane. After being blocked with 5% skim milk (Becton, Dickinson and Company) in TBST for 1 hr at room temperature, the membranes were incubated with respective I^{ry} antibodies of A-Raf, B-Raf, C-Raf (Santa Cruz), MEK1/2 (Cell Signaling), phospho ERK1/2, ERK1/2 (Cell Signaling), and GAPDH (AbFrontier) overnight at 4 ℃. After incubating the membranes with Goat anti rabbit IgG (Santa Cruz) or Goat anti mouse IgG (Santa Cruz) for 1 hrs at room temperature, proteins were visualized using the enhanced chemiluminescence (Advansta) solution as described by the manufacturer.

10. Statistical analysis

Data were presented as the means +/- standard deviation. Results were analysed using one-way ANOVA with SPSS version 21.

III. RESULTS

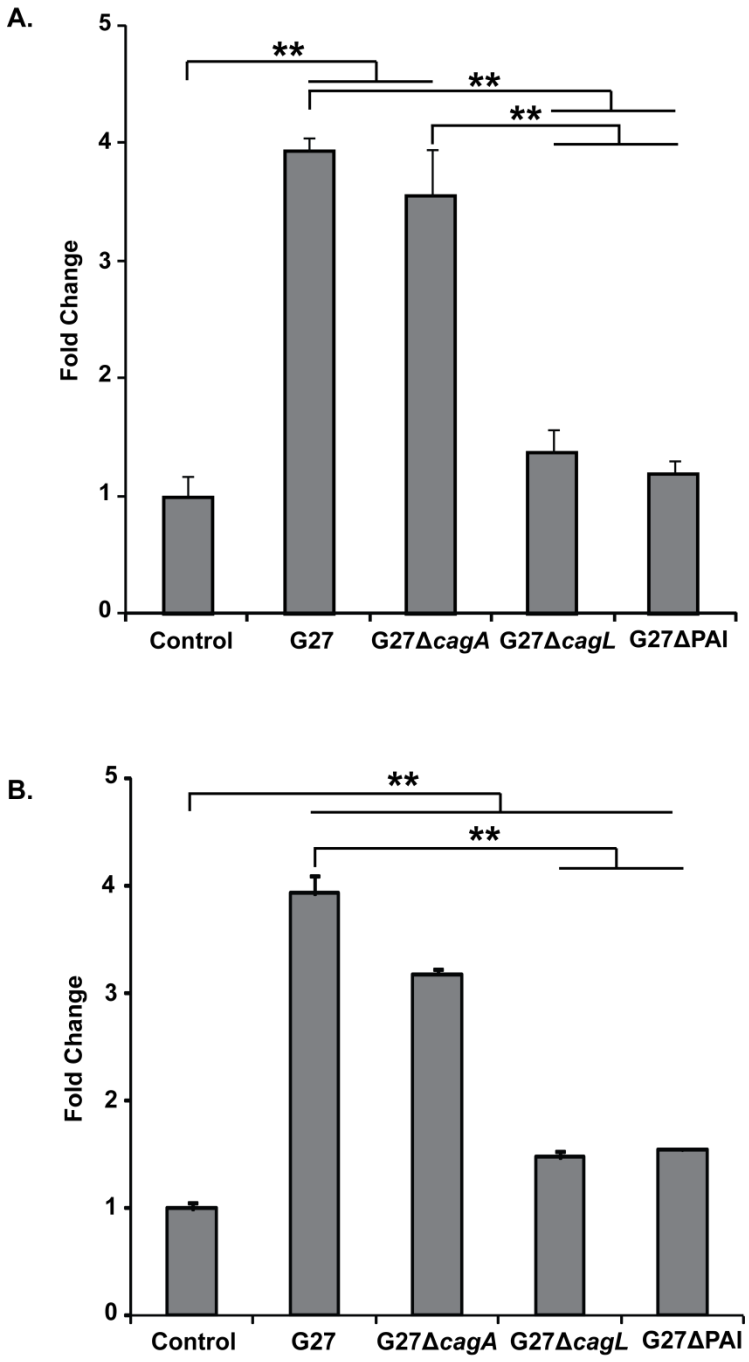
1. *H. pylori* infection induces gastrin promoter activity

To examine the ability of *H. pylori* to regulate gastrin expression, we constructed stable AGS cells expressing gastrin promoter-luciferase reporter (G240-Luc) and stimulated the cells with *H. pylori* G27 WT and its isogenic mutants (Figure 2).

H. pylori strain G27 WT, which possesses functional *cag*PAI induces the gastrin promoter about four fold compared to the promoter activity level expressed by uninfected, control G240-Luc cells. In addition, *H. pylori* G27Δ*cag*A, which lacks the major virulence factor CagA, showed enhanced promoter induction, which was in similar level to the promoter induction by wild type *H. pylori*.

In contrast, *H. pylori* G27Δ*cagL* which lacks *cagL* and *H. pylori* G27ΔPAI lacking the entire *cag*PAI, which is responsible for the formation of T4SS, showed a significant reduction in gastrin promoter activation.

This result suggests that, *H. pylori* harboring functional T4SS activates the gastrin promoter and the promoter activation is independent of CagA. Furthermore, this result confirms that, constructed G240-Luc cells can be used for further experiments to explore gastrin promoter activity regulates by various stimulants.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G27ΔcagL
G27ΔPAI

Figure 2. Gastrin promoter stimulation by *H. pylori* G27 wild type and its isogenic mutant strains.

Stable G240-Luc cells (A) and G1.3-Luc cells (B) were placed in serum free media for 24 hrs and then stimulated with *H. pylori* G27 wild type and its isogenic mutants; G27Δ*cagA*,G27Δ*cagL* and G27ΔPAI. Five hours after infection, cells were lysed and luciferase activity was measured. Fold changes were the ratio of the stimulated Gas-Luc luciferase activity compared to basal luciferase activity of unstimulated cells. The mean fold change ± SD, for four separate experiments which performed in triplicate is shown. ** p <0.001.

2. Effect of *H. pylori* multiplicity of infection (MOI) on gastrin promoter expression.

In order to find out whether the gastrin promoter induction by *H. pylori* is depend on the number of bacteria to cells during infection, also referred to as multiplicity of infection (MOI), the G240-Luc cells were infected with MOI 100 and 200. MOI 100 was frequently utilized to study the effect of *H. pylori* infection on the host cellular signaling pathways hence, it was selected here too. As shown in Figure 3, G240-Luc cells infected with MOI 100 and 200 showed significant promoter activation compared to control cells and there were no significant differences between the gastrin promoter activation by *H. pylori* strain G27 MOI 100 and 200. So, MOI 100 was selected to perform all following experiments.

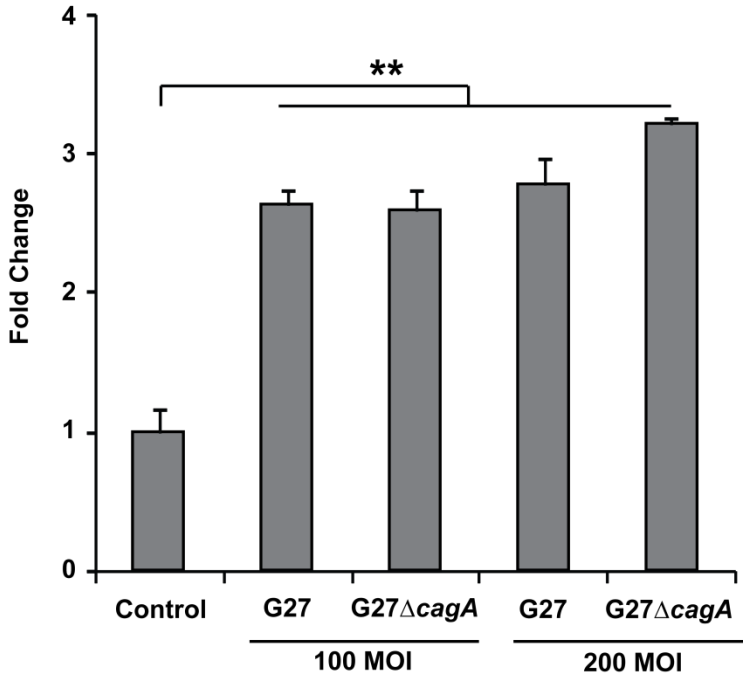
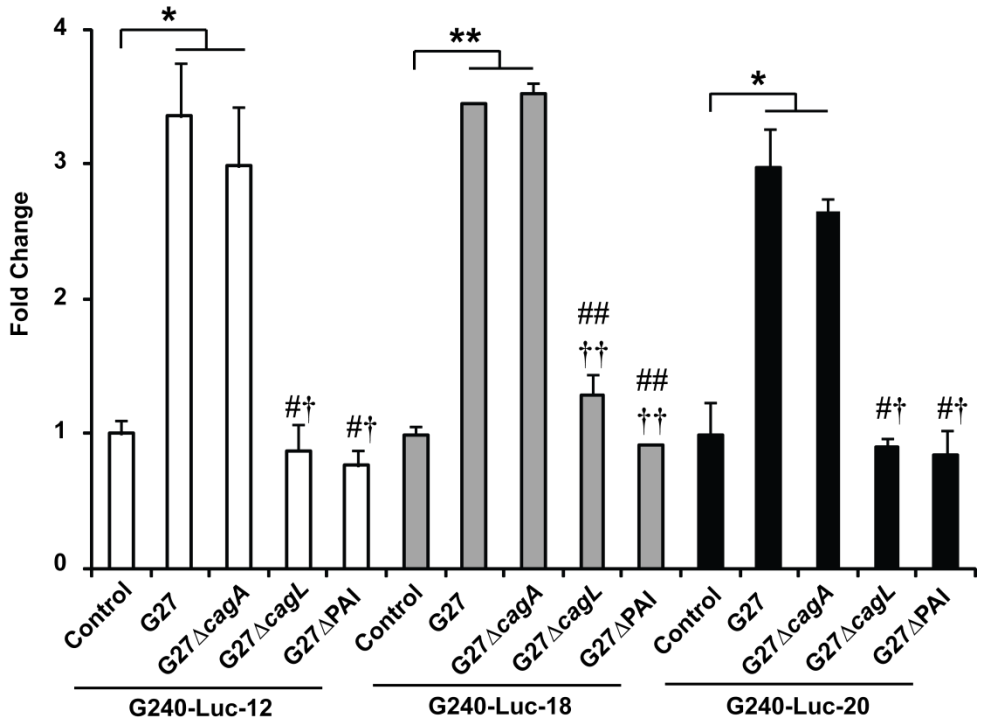


Figure 3. Effect of *H. pylori* multiplicity of infection (MOI) on gastrin expression

G240-Luc stable AGS cells were infected with G27 (WT) and, G27Δ*cagA* strains in MOI 100 and 200. Luciferase expression level has shown as the fold change compared to control cells expression values. ** p <0.001

3. *H. pylori*-induced gastrin promoter activity on different pools of stable transformants.

To examine the *H. pylori*-induced gastrin promoter activity in different pools of stable transformants, G240-Luc cells were selected and pooled as described in the methods section. Several pools of stable transformants with high luciferase expression levels were cultured and infected with *H. pylori* to check the gastrin promoter activity in those cells. Figure 4 shows the gastrin promoter activation by *H. pylori* strain G27 WT and its isogenic mutants in 3 different pools of stable transformants named as G240-Luc-12, G240-Luc-18 and G240-Luc-20. All three pools of stable transformants showed a similar pattern of promoter activation with *H. pylori* suggesting that, this *H. pylori* effect on gastrin expression is not occurring only in some pool of stable cells, but it's a generalized activity that can be seen in several transformants.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Control
G27
G27ΔcagA
G27ΔcagL
G27ΔPAI
G240-Luc-12
G240-Luc-18
G240-Luc-20

Figure 4. *H. pylori*-induced gastrin promoter activity on different pools of stable transformants.

Several pools of stable transformants of G240-Luc cells (named as G240-Luc-12, G240-Luc-18, G240-Luc-20) were selected and stimulated with *H. pylori* strain G27 (WT) and its isogenic mutants G27Δ*cagA*, G27Δ*cagL*, G27ΔPAI for 5 hrs. Then cells were lysed and luciferase assay was performed. Normalized luciferase activity levels have shown as relative fold changes compared to unstimulated control cells of respective group. The data show the mean ±SD of fold change from three experiments. * and ** indicate significant differences of G27 and G27Δ*cagA* compared to control at $p \leq 0.05$, $p \leq 0.001$, respectively. # and † indicate significant differences compared to G27 and G27Δ*cagA* respectively ($p \leq 0.05$). ## and †† indicate significant differences compared to G27 and G27Δ*cagA* respectively at $p \leq 0.001$.

4. Gastrin promoter activation by different *H. pylori* strains.

In *H. pylori* infected patients, there is variability in the clinical outcome that is associated with genetic variation of *H. pylori* strains. Because of that, we examined the differences in the gastric promoter induction by different *H. pylori* strains. G240-Luc cells were co-cultured with *H. pylori* 26695 (WT), which is known as a western type of *H. pylori* and *H. pylori* K74 (WT), which is a clinical Korean isolate. Both of those western and East Asian *H. pylori* strains can activate gastrin promoter for about 4 folds compared to control stable cells (Figure 5). Additionally, G240-Luc cells co-cultured with *H. pylori* strain K74Δ*cagL*, didn't show any significant promoter induction. On the other hand complementation of *H. pylori cagL* deletion mutants with K74*cagL*, restored the gastrin promoter activity similar to WT *H. pylori*-stimulated promoter induction. This result suggests that, different *H. pylori* strains also can induce the gastrin promoter activity significantly.

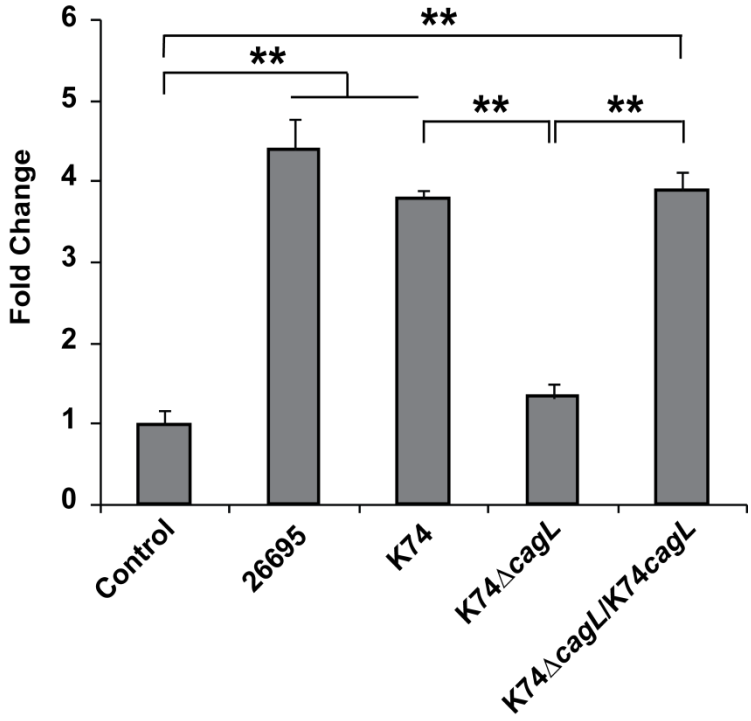


Figure 5. Gastrin promoter stimulation by different *H. pylori* strains

G240-Luc cells were stimulated with *H. pylori* strains 26695, K74 or K74 isogenic mutant K74Δ*cagL* and K74Δ*cagL* complemented with K74*cagL* (K74Δ*cagL*/ K74*cagL*) for 5 hrs. After infection, cells were lysed and luciferase assay was performed. Normalized luciferase expression levels in relation to uninfected control cells were plotted. The mean values ± SD of at least three independent experiments performed in triplicates are shown. ** $p \leq 0.001$.

5. Induction of gastrin promoter requires EGFR activation.

Phosphorylation of EGF receptor is known to activate the Ras/Raf/MEK pathway resulting in the activation of ERK 1/2. EGF has been reported as an important molecule in the stimulation of gastrin promoter activity via EGFR [47]. To examine the importance of EGFR activation in *H. pylori*-stimulated gastrin expression, G240-Luc cells were pre-incubated with the EGF receptor inhibitor, AG1478 (1 μM) for 30 min and stimulated with *H. pylori* strain G27. As shown in Figure 6, AG1478 treatment reduced the *H. pylori*-stimulated G240-Luc reporter activity significantly compared to unstimulated G240-Luc cells. Even though, there was a significant reduction in the *H. pylori*-induced gastrin promoter activity with AG1478, still higher level of promoter activity is remained ($p \leq 0.05$). This result suggests that EGFR kinase activity is important in *H. pylori*-induced gastrin expression, but the promoter activity is not solely depend on EGFR activation.

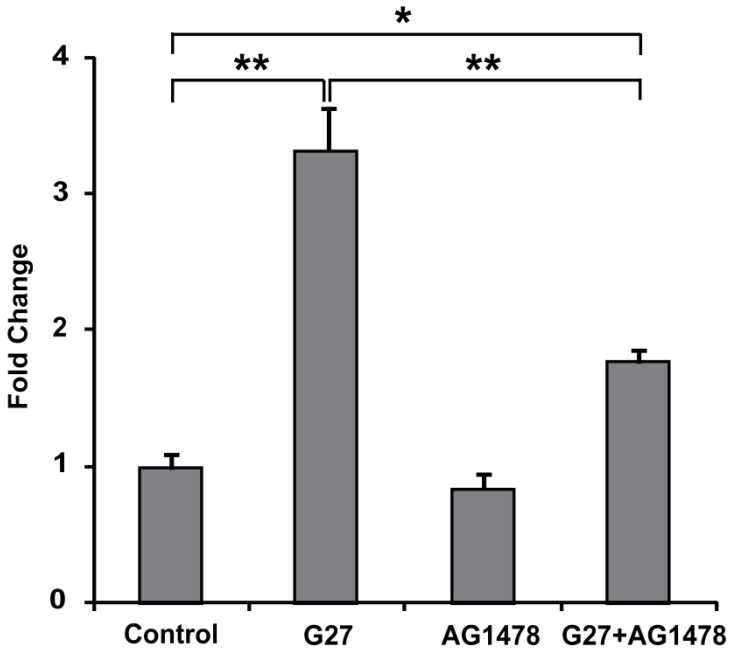


Figure 6. *H. pylori*-induced gastrin promoter activity is inhibited in the presence of EGFR kinase inhibitor.

G240-Luc cells were preincubated with AG1478 (1 µM) and stimulated with *H. pylori* strain G27. Luciferase activities in relation to unstimulated cells were plotted. The mean values of ± SD of at least three independent experiments performed in triplicate are shown. * $p \leq 0.05$, ** $p \leq 0.001$

6. EGFR activation is needed for HB-EGF-stimulated gastrin promoter activity.

Previous studies have demonstrated that, *H. pylori* can up regulate and induce both HB-EGF gene expression and ectodomain shedding in human gastric cells [70,71]. Furthermore, it has been shown that mature HB-EGF activates EGFR, by tyrosine phosphorylation. We therefore examined whether HB-EGF can induce gastrin promote activity. The G240-Luc cells were stimulated with 10 nM HB-EGF along with 10 nM EGF as a positive control. As shown in Figure 7A, HB-EGF showed an induction of gastrin promoter activity in a similar level to the EGF-mediated gastrin promoter activation.

The EGFR activation is dependent on the extracellular transmembrane metalloprotease cleavage of pro HB-EGF and signaling by mature HB-EGF [71]. In order to identify the importance of EGFR activation in HB-EGF-stimulated gastrin expression, the G240-Luc cells were pre-treated with AG1478 followed by 10 nM of HB-EGF treatment. As shown in Figure 7B, AG1478 treatment significantly reduced the HB-EGF-stimulated gastrin promoter activity, confirming that EGFR activation is important in HB-EGF stimulated gastrin promoter activity.

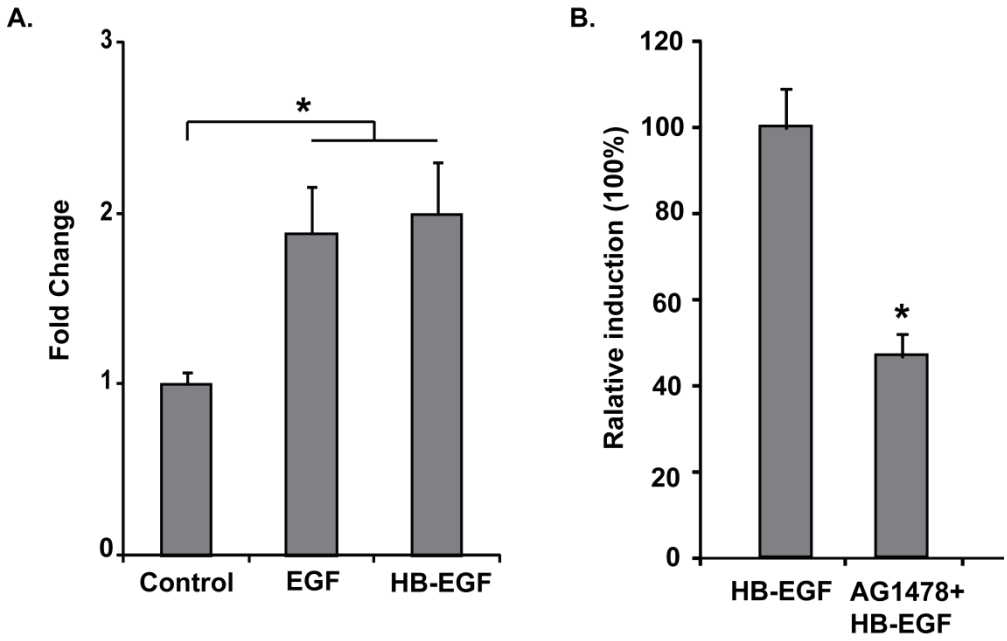


Figure 7. HB-EGF can stimulate the gastrin promoter activity

A. Serum starved G240-Luc cells were stimulated with 10 nM of EGF or HB-EGF for 5 hrs. Luciferase activities were measured and reported as fold increase compared with unstimulated control. Results are expressed as mean ± SD of three independent experiments performed in triplicates. * $p \leq 0.05$.

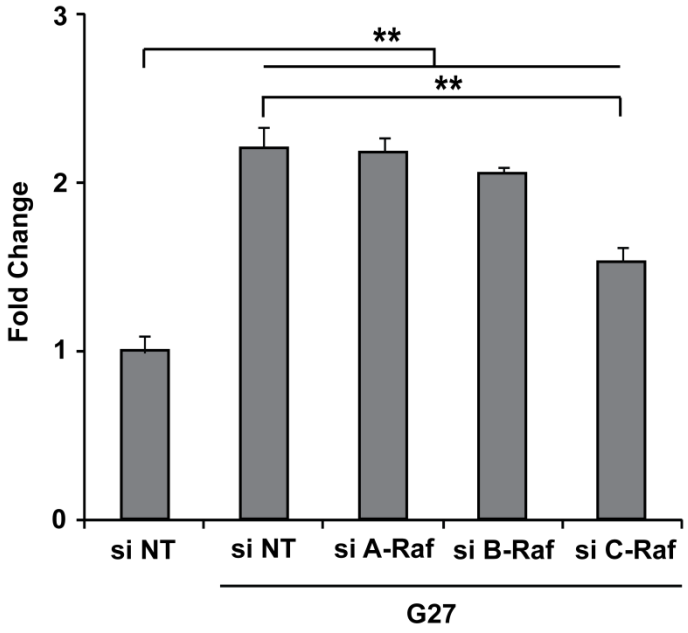
B. Serum starved G240-Luc cells were pre-treated with control (DMSO) or EGFR kinase inhibitor AG1478 (1 μM) for 30 min and then stimulated with HB-EGF (10 nM) for 5 hrs. Luciferase activity was measured and reported as a percentage relative to control (DMSO treated) G240-Luc cells. The results are expressed as a mean ± SD of three independent experiments performed in triplicates. ** $p \leq 0.05$.

7. C-Raf is important in *H. pylori*-induced gastrin gene expression.

To further delineate the mechanism leading to gastrin promoter induction by *H. pylori*, we investigated the involvement of Raf isoforms in gastrin promoter activity. G240-Luc cells were transfected with A, B or C-Raf siRNA followed by *H. pylori* infection and then luciferase expression levels were measured.

As shown in Figure 8A, a significant reduction of promoter activity was observed with C-Raf siRNA treatment. On the other hand, A-Raf and B-Raf siRNA treatments didn't show any reduction in the *H. pylori*-induced gastrin promoter activity. To verify the knockdown of protein expression by siRNA treatment, western blot was performed (Figure 8B). This result suggests that C-Raf is important in *H. pylori*-induced gastrin expression.

A.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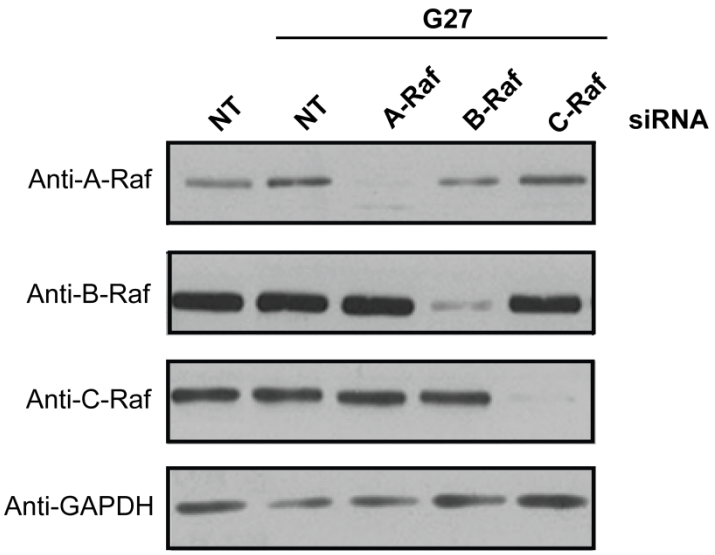
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Anti-C-Raf
Anti-GAPDH

Figure 8. C-Raf is important in *H. pylori*-induced gastrin promoter activation.

A. G240-Luc cells were transfected with Non-targeting siRNA (si NT), A-Raf siRNA (si A-Raf), B-Raf siRNA (si B-Raf) or C-Raf siRNA (si C-Raf). Non-targeting siRNA was used as knockdown negative control. After 48 hrs, cells were stimulated with *H. pylori* strain G27 for 5 hrs. Luciferase assay was carried out, and the results are reported as fold change with respect to the NT siRNA. Triplicates ± SD are shown. ** $p \leq 0.001$.

B. Western blot analyses were performed to confirm A-Raf, B-Raf or C-Raf knockdown on the protein level. GAPDH served as a loading control.

8. MEK1 plays a major role in *H. pylori*-induced gastrin promoter activity.

Activated Raf phosphorylates its downstream substrate MEK (Mitogen activated protein kinase). Therefore, the effect of MEK1 and MEK2 on the gastrin gene expression by *H. pylori* was examined in this part of the experiment. In order to knockdown MEK1 and MEK2 activities G240-Luc cells were treated with MEK1 and MEK2 siRNA alone or together. Interestingly, there was a significant reduction of luciferase activity with MEK1 siRNA treatment, while MEK2 knockdown didn't show any significant reduction of the luciferase activity compared to the luciferase activities of non-targeting siRNA treated G240-Luc cells (Figure 8A). Furthermore, the cells treated with MEK1 and MEK2 siRNA combination (MEK1/2) also showed a significant reduction of gastrin promoter activity. Western blotting was carried out to confirm the MEK1 and MEK2 knockdown by siRNA (Figure 9B). This result can be concluded as MEK1 plays a major role in *H. pylori*-induced gastrin promoter induction, while the role played by MEK2 is dispensable.

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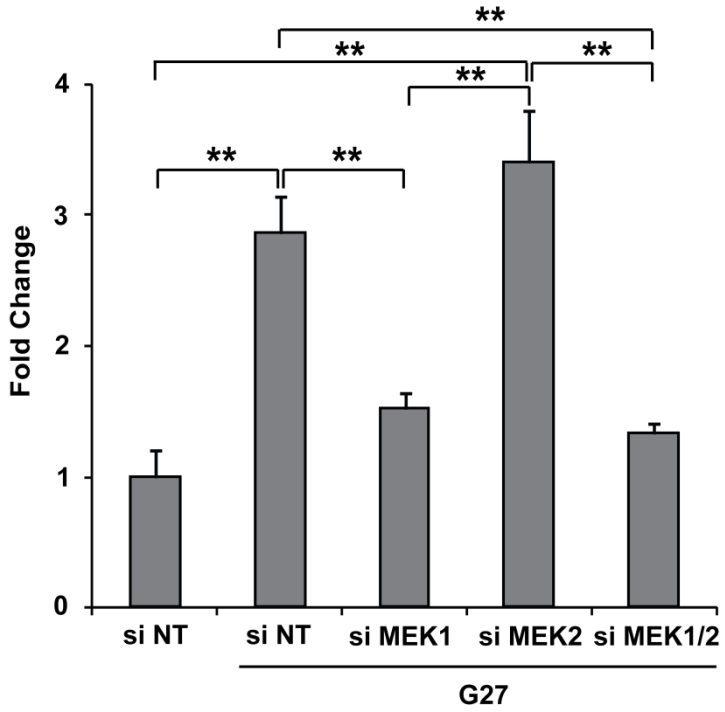
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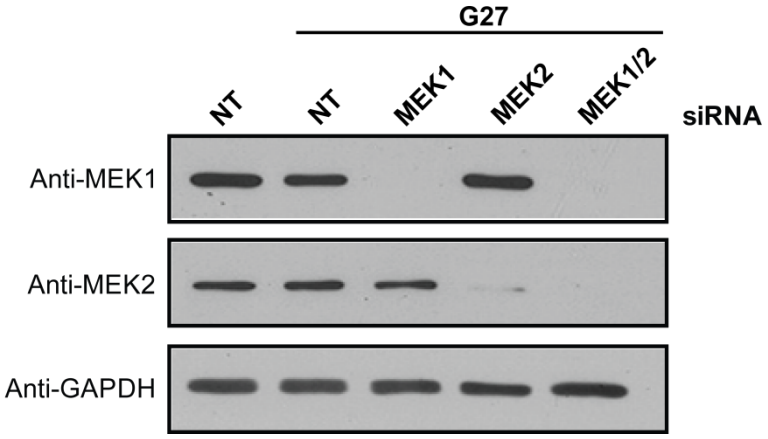
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Anti-GAPDH

Figure 9. MEK1 is important in *H. pylori*-induced gastrin promoter activation.

(A). G240-Luc cells were transfected with non-targeting siRNA (si NT), MEK1 siRNA (si MEK1), MEK2 siRNA (si MEK2), MEK1 siRNA+MEK2 siRNA (si MEK1/2) (40 pmol/ ml) and after 48 hrs, cells were co-cultured with *H. pylori* G27 strain (G27) for 5 hrs prior to lysis of cells for luciferase assay. Normalized luciferase expression values are expressed as fold induction compared with uninfected G240-Luc cells treated with non-targeting siRNA alone. Western blot analysis was performed to confirm MEK1 and MEK2 knockdown on protein level (B). GAPDH used as a loading control. Mean ± SD of four independent experiments performed in triplicates are shown. ** $p \leq 0.001$ was considered statistically significant.

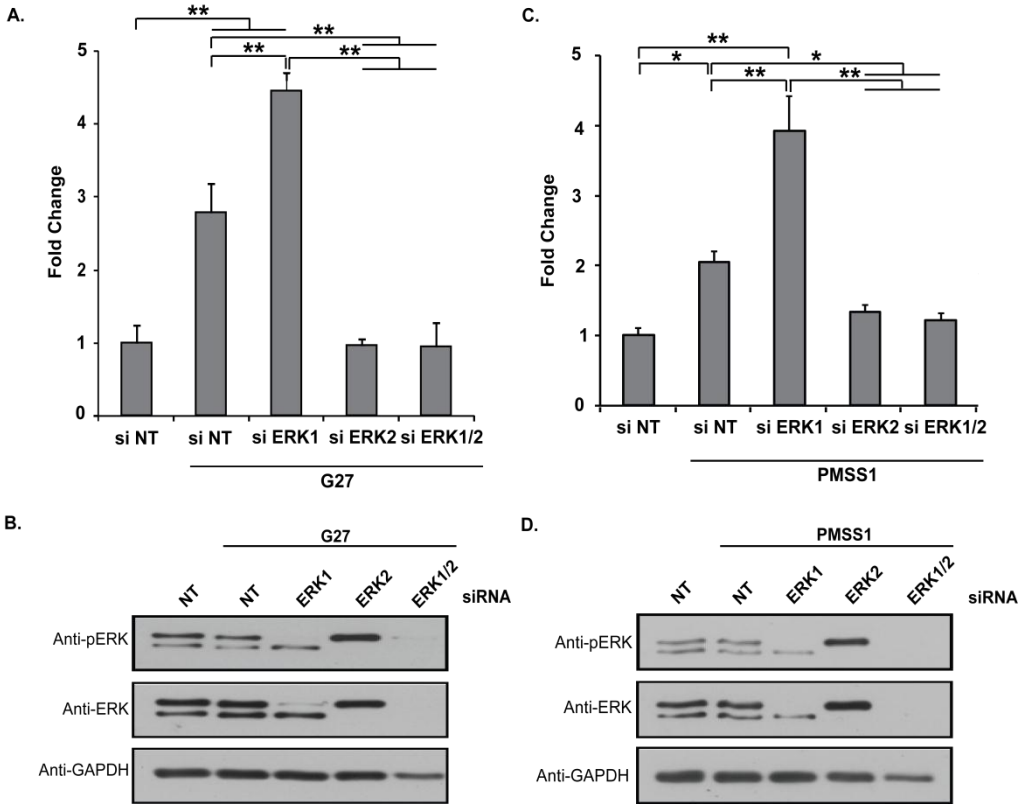
9. ERK1 and ERK2 involve differently on *H. pylori*-induced gastrin promoter activity.

ERK is the downstream target of MEK and it was interesting to see the effect of ERK silencing on the gastrin expression. So the involvement of ERK1 and ERK2 on gastrin promoter stimulation by *H. pylori* was examined in this part of the study. G240-Luc cells were transfected with ERK1 and ERK2 siRNA and subsequently stimulated with *H. pylori* G27 WT. Intriguingly, with ERK1 siRNA treatment, significant induction of gastrin promoter activity was observed, compared to non-targeting siRNA treated cells (Figure 10A). In contrast to that, the ERK2 siRNA treatment showed a significant reduction of gastrin promoter activity. Moreover, combined treatment of ERK1 and ERK2 siRNA also significantly reduced the *H. pylori* G27 strain-stimulated gastrin promoter activity.

As this is an interesting result, to exclude the *H. pylori* strain specificity on this ERK activation, we stimulated G240-Luc cells with mouse adapted *H. pylori* strain PMSS1. As shown in figure 10C, PMSS1-stimulated gastrin promoter activity also showed a similar pattern to G27-induced gastrin promoter activity excluding the strain specificity of this interesting result.

H. pylori CagA-induced MAPK pathway can be divergent from the classic MAPK pathway. Even though, there's no difference in the gastrin promoter induction by *H. pylori* G27Δ*cagA* compared to G27 WT, it's interesting to

examine the ERK modulation in G27Δ*cagA*-induced gastrin expression. Hence, G240-Luc cells were transfected with ERK siRNA and stimulated with *H. pylori* G27Δ*cagA* strain. As observed with G27 WT, *H. pylori* G27Δ*cagA* strain also showed significant induction of gastrin promoter activity with ERK1 siRNA treatment and significant reduction of promoter activity with ERK2 siRNA treatment (Figure 10E). This finding suggests that ERK1 and ERK2 play different roles in *H. pylori*-stimulated gastrin promoter induction and also these different roles of ERK1 and ERK2 in gastrin promoter expression is independent of *H. pylori* major virulence factor CagA.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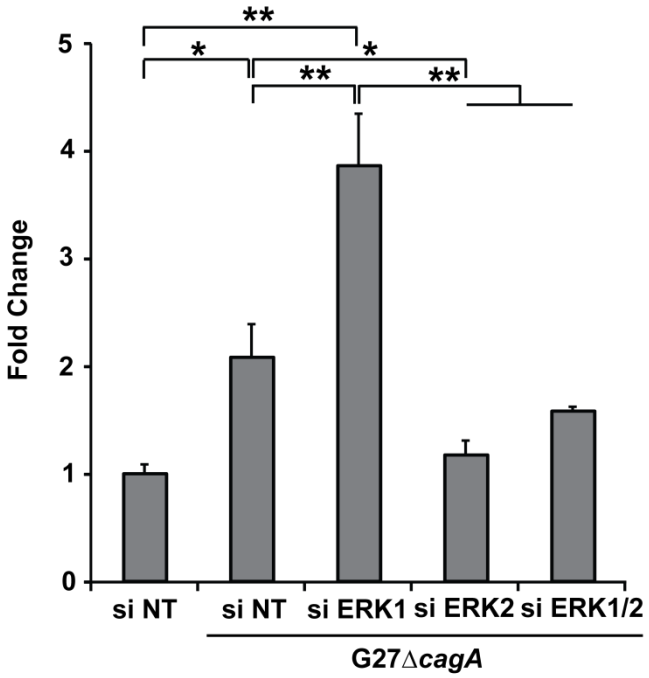
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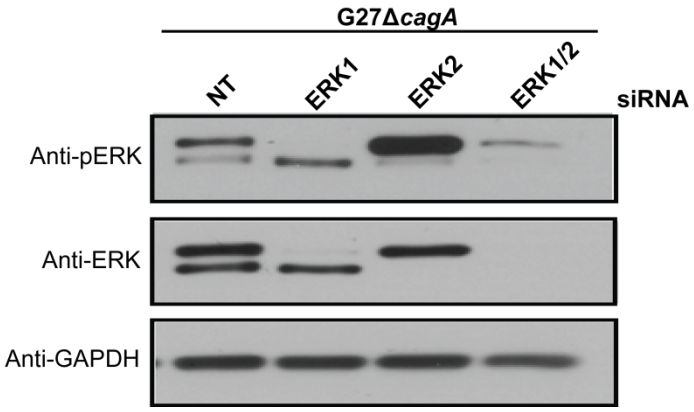
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Anti-GAPDH

Figure 10. Roles of ERK1 and ERK2 in *H. pylori*-induced gastrin promoter activity

G240-Luc cells were transfected with 40 pmol/ml of non-targeting siRNA (si NT), ERK1 siRNA (si ERK1), ERK2 siRNA (si ERK2) separately or ERK1/ERK2 siRNA together (si ERK1/2). After 48 hrs, the cells were co-cultured with *H. pylori* strains G27 WT (A), *H. pylori* PMSS1 WT (C) or G27Δ*cagA* (E). After 5 hrs of infection, cells were lysed, luciferase activity and protein concentration in cell lysates were determined and normalized. Normalized luciferase expression values in relation to *H. pylori* G27 WT (A), PMSS1 (C) or G27Δ*cagA* (E) have shown as the fold changes compared to basal luciferase expression of uninfected, non-targeting siRNA treated cells. Western blot analyses of G240-Luc cells treated with NT or ERK1, ERK2, ERK1/2 siRNA followed by the infection of *H. pylori* G27 (B), PMSS1 (D), G27Δ*cagA* (F) were performed to detect knockdown of ERK1 or ERK2. Total cell lysates were subjected to western blot analysis with anti-phospho ERK1/2 (pERK), anti-total ERK1/2 and anti-GAPDH antibodies. Non-targeting siRNA served as knockdown negative control and GAPDH served as the loading control. Data are expressed as mean ± SD of four independent experiments. * $p \leq 0.05$, ** $p \leq 0.001$.

IV. DISCUSSION

The results of this study demonstrated that *H. pylori* stimulates gastrin promoter activity via the MAPK pathway signal molecules C-Raf, MEK1 and ERK2. Our data demonstrated for the first time, that ERK2 plays a vital role in *H. pylori*-induced gastrin expression. In addition to that, we have shown that HB-EGF is a novel growth factor which possesses the ability to induce the gastrin promoter activity besides the EGF, which is the only known EGF family growth factor having this ability so far.

H. pylori activates MAP kinases after contact with gastric epithelial cells [58,72]. A number of bacterial factors have been involved in MAPK activation including *H. pylori* CagA. However, it's clear that *H. pylori* T4SS is the major factor of MAPK activation [58,72]. In addition, it's a well known fact that *H. pylori* is associated with hypergastrinemia, which has been observed in various studies conducted in human subjects as well as in *in vitro* and *in vivo*. In this study, we found that functional T4SS is important in *H. pylori*-induced gastrin expression. This is in agreement with other studies which have been shown that the *H. pylori cag*PAI is associated with hypergastrinemia. [73]. This will further lead to think about the T4SS factor which is responsible for the promoter induction. Even though the involvement of CagA in gastrin expression is controversial, [47,60,61] in agreement with majority of recent studies, [47,60] this study also revealed that the gastrin promoter activation is

independent of CagA. In addition to that, a recent study has reported the *H. pylori* CagL as a novel ligand activating the gastrin promoter [60]. However, it was difficult to rule out the importance of the CagL alone in this study as deletion of *cagL* impairs assembly of type IV secretion system.

Infection of gastric epithelial cells with *H. pylori* has been shown to result in increased HB-EGF gene transcription *in vitro* and *in vivo* [70,71,74]. Both HB-EGF gene expression and protein shedding increased in *H. pylori* infection [71,75]. Not only that, gastrin also acts on CCK-2 receptors to induce the HB-EGF gene expression and ectodomain shedding [76]. Moreover, HB-EGF was significantly up-regulated in the gastric mucosa of hypergastrinemic mice during the premalignant stages of cancer development [43]. As *H. pylori* infection and gastrin both can induce the HB-EGF gene expression, it was raised our curiosity to investigate the effect of HB-EGF on gastrin promoter activity which has not been addressed so far.

As a result, this study revealed that HB-EGF also can activate gastrin promoter. So far EGF was known as the only EGF growth factor family member which is having the ability to induce the gastrin expression [20,62,77]. But this study will add another EGF family member as a gastrin promoter activator.

Furthermore, our results showed that, HB-EGF-stimulated gastrin promoter activity is dependent on EGFR activation. In previous studies, HB-EGF was

determined to be the ligand that mediates *H. pylori*-induced EGFR transactivation [71,78]. Hence we can assume that, activation of EGFR via HB-EGF may play a significant role in *H. pylori*-induced gastrin expression.

As MAPK pathway is the extensively studied signal pathway, several studies have focused on MAPK pathway involvement in gastrin promoter activation and gastrin expression. With regards to EGF, it has been shown that EGF induces gastrin promoter via this pathway [62]. In addition, few studies conducted to explore the signal pathways in *H. pylori*-stimulated gastrin promoter activation using kinase inhibitors, revealed the involvement of MAPK pathway in gastrin expression [47,60].

But our study used siRNA to knockdown MAPK pathway signal isoforms and identified the importance and the contribution of each kinase isoform clearly in the *H .pylori*-stimulated gastrin promoter activity.

After finding out that, EGFR activation by *H. pylori* is important in gastrin promoter activation, we examined the involvement of A-Raf, B-Raf and C-Raf in *H. pylori*-stimulated gastrin promoter activity. This study demonstrated for the first time that the contribution of A-Raf and B-Raf is dispensable in gastrin promoter activation, while C-Raf plays an important role.

We couldn't address the importance of the Ras activation in this experiment. But *in vitro* experiments using several gastric cancer cell lines have been revealed that oncogenic Ras activates gastrin promoter in gastric cancer cells,

suggesting the importance of Ras signal molecule in gastrin expression [79]. So Ras activation may lie upstream of the Raf, and from that Ras kinase activity C-Raf may be activated.

One important aspect of the regulation of the Ras-ERK cascade is the specific, non-redundant roles of protein isoforms in this pathway. From our study, we conclude that MEK1 plays an imperative role in *H. pylori*-stimulated gastrin promoter induction than MEK2. MEK1 and MEK2 isoforms are highly homologous, being 80% similar in the overall sequence and 90% similar in their kinase domain [80]. But non-redundant functions of MEK1 and MEK2 have been identified in cell cycle regulation, carcinogenesis and development [81-84]. Our finding of MEK1 involvement in *H. pylori*-stimulated gastrin promoter activity will further support these non-redundant functions of MEK isoforms.

Experiments carried out in the present study to examine the contribution of ERK isoforms in *H. pylori*-induced gastrin promoter activity revealed an unexpected relationship between ERK1 and ERK2. ERK1 knockdown showed significant induction of gastrin promoter activity, while ERK2 knockdown showed significant reduction of promoter activity. ERK1 and ERK2 have a higher sequence identity and participate equally in many cellular functions [54]. They are coexpressed in most tissues with different abundance which is thought to contribute for the differential functions of these two isoforms [85].

Results from *in vivo* experiments using ERK knockout mice provide convincing evidences of the distinct functions of ERK isoforms. One study showed that ERK2-deficient mice die early in development showing that ERK1 cannot compensate in the embryo for ERK2 [86], while another one showed that ERK1-deficient mice are viable, with no obvious compensatory upregulation of ERK2 protein levels but showed minor defects such as deficit in thymocyte maturation [87]. Not only that an *in vitro* study which has used mouse embryo fibroblasts and NIH 3T3 cells showed ablation of ERK1 results in an enhancement of ERK2-dependent signaling and in a significant growth advantage. By contrast, knockdown of ERK2 almost completely abolishes normal and Ras-dependent cell proliferation [88].

Our results of ERK siRNA treatment on gastrin promoter activity also suggest that ERK2 function is indispensable and inducible regards the gastrin expression. On the other hand ERK2 knockdown significantly reduced the gastrin promoter activity, suggesting that the ERK1 function is controlling the ERK2 activation. There can be various possibilities which can give rise this result. One possible explanation is that ERK1 competes with ERK2 for the upstream MEK [89]. If this is true in this study, the gastrin promoter activity is mediated solely by ERK2 homodimers and ERK1 homodimers alone may not activate the gastrin promoter. In addition to that, this will give rise another question whether ERK1 and ERK2 activate different transcription factors

which are having promoter repression activity or promoter inducing activity respectively. Previously it has been identified that Sp1 transcription factor is important in EGF-mediated and also *H. pylori*-mediated gastrin expression [62]. But further studies are needed to understand the distinct roles of ERK isoforms in *H. pylori*-stimulated gastrin expression. It will be important to explore further the mechanism underlying the negative role played by ERK1 as this can be useful as a therapeutic target to lessen the *H. pylori*-stimulated hypergastrinemia.

Taken together, this current study demonstrates that *H. pylori* which are having functional T4SS induce the gastrin promoter via EGFR, C-Raf, MEK1 and ERK2 in MAPK pathway. This study is first to show that, ERK2 plays an inducible role, while ERK1 plays an inhibitory role in the context of *H. pylori*-stimulated gastrin expression. Furthermore, this study reveals HB-EGF also can activate the gastrin promoter hence speculates that *H. pylori* activation of EGFR via HB-EGF may play a role in the gastrin expression stimulated by *H. pylori* (Figure 11). The understandings of the involvement of MAPK pathway will help to develop therapeutic strategies to reduce *H. pylori*-induced hypergastrinemia consequently development of gastric cancers.

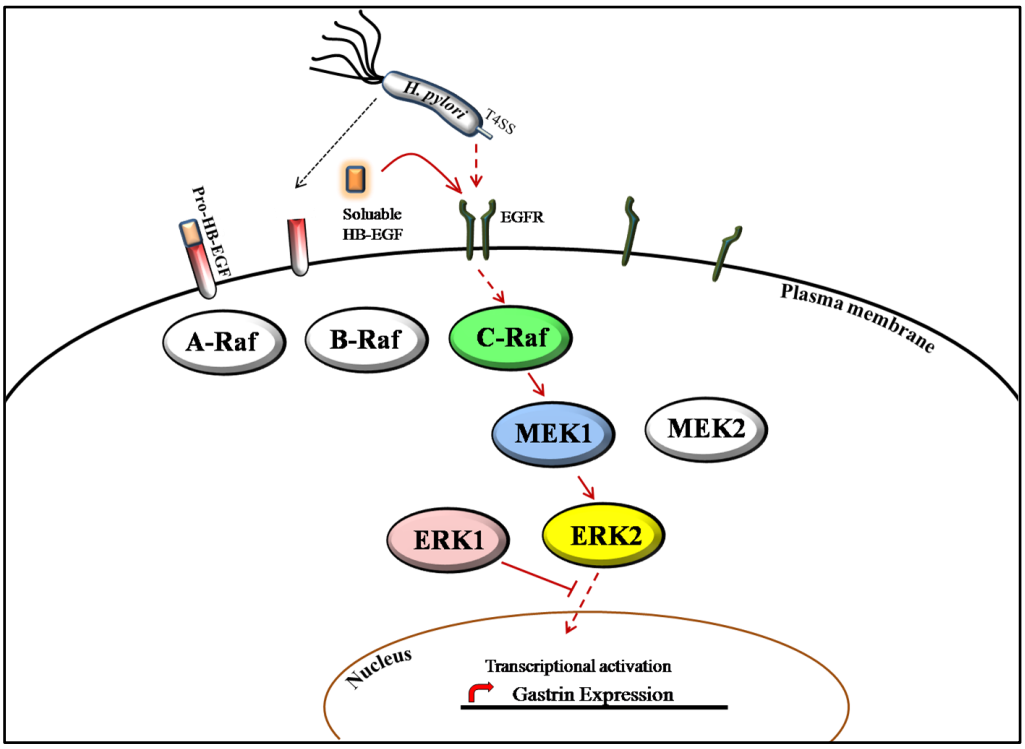

Figure 11. Schematic diagram of ERK signal pathway involvement in *H. pylori*-induced gastrin promoter activation.

H. pylori with type IV secretion system, activates EGF receptors (EGFR), which in turn facilitates activation of C-Raf, MEK1 and ERK2 to induce the gastrin promoter. ERK1 may play an inhibitory role in this promoter activation, possibly exerting an inhibitory effect on ERK2. Both HB-EGF gene expression and protein shedding are increased in *H. pylori* infection. HB-EGF also can induce the gastrin promoter activity and EGFR activation via HB-EGF may play a role in *H. pylori*-induced gastrin expression.

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국문요약

헬리코박터 파일로리가 활성화 시키는 가스트린 발현에서의 C-Raf/MEK1/ERK2의 역할

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위 속 산성 환경에서 서식할 수 있는 그람 음성 세균인 헬리코박터 파일로리는 다양한 위장질환의 주요 유발인자이다. 헬리코박터에 감염된 환자들은 가스트린 분비의 증가로 인하여 고가스트린 혈증 (hypergastrinemia)이 유도되고 이 상태가 장기간 지속되면 만성 위염과 위축성 위염으로 발전하게 된다. 가스트린은 위산 분비를 조절하는 매주 중요한 호르몬으로 알려져 있고, 위 상피세포의 성장과 분화 과정에 밀접하게 관련되어 있는 것으로 보고되어 있다. 헬리코박터 감염에 의해 유도되는 고가스트린 혈증은 위암으로 발전하는 하나의 요인으로 작용한다. MAPK (mitogen activated protein kinase) pathway는

가스트린 발현을 조절하는 매우 중요한 세포 신호전달 과정이다. 헬리코박터 감염은 숙주세포의 MAPK pathway를 교란시켜 비정상적인 세포 신호전달을 유도한다고 보고되어 있다. 그러나 헬리코박터 감염에 의한 MAPK pathway의 변화가 가스트린 발현증가에 어떻게 관여하는지는 정확히 밝혀져 있지 않다. 본 연구는 헬리코박터 감염에 의해 유도되는 가스트린 발현과 관련된 기전을 MAPK pathway를 중심으로 자세히 조사하였다.

가스트린 promotor의 발현을 평가하기 위하여 사람 위암 세포주 (AGS cell)에 gastrin promoter-luciferase reporter를 stable transfection 시킨 G240-Luc 세포를 이용하여 gastrin promoter의 활성을 측정하였다. 가스트린 발현을 유도하는 헬리코박터 구성 성분이 무엇인지 알아보기 위하여 헬리코박터 균주 G27 wild type과 G27Δ*cagA*, G27Δ*cagL* 그리고 G27ΔPAI와 같은 isogenic mutants (동종 변이균주)를 만들어 각 실험에 이용하였다. G27Δ*cagA* 균주는 헬리코박터의 제 4형 분비기관에 의해 숙주세포 내로 주입되는 독성인자인 *cagA* 유전자가, G27Δ*cagL* 균주는 헬리코박터의 제4형 분비기관을 구성하고 있는 단백질들 중 하나인 *cagL* 유전자가 결손되어 있는 변이 균주이다. G27ΔPAI 균주는 제 4형 분비기관을 encoding 하고 있는 *cag* pathogenicity island 유전자 전체가 결손되어 있다. 헬리코박터 G27 wild type 혹은 그 동종 변이균주들을 각각 G240-Luc 세포주에 처리한 후 luciferase 발현을 측정하였다. 가스트린 발현을 평가하기 위해 양성 대조군으로 성장인자 EGF를 많이 사용하는데 이와 유사한 HB-EGF 성장인자는 가스트린 발현에 어떠한 영향을 끼치는지 알기 위해서 HB-EGF를

G240-Luc 세포주에 처리하였다. MAPK pathway를 구성하고 있는 단백질 Raf family (A-Raf, B-Raf, C-Raf)와 MEK family (MEK1, MEK2), 그리고 ERK family (ERK1, ERK2)에 대한 각각의 siRNA를 제작하여 처리하였다. 각 단백질 발현 억제 정도는 western blotting 방법을 이용하여 확인하였다.

다양한 동종 변이체들중 G27 wild type 과 G27Δ*cagA* 균주를 G240-Luc 세포주에 감염시켰을 때 가스트린 promoter 활성이 강하게 증가하였다. 반면 G27Δ*cagL* 혹은 G27ΔPAI 균주는 가스트린 promoter 활성을 유도하지 않았다. 이 결과는 헬리코박터 제 4형 분비기관이 가스트린 promoter 활성에 중요하게 관여할 수 있다는 것을 제시한다. 또한 HB-EGF를 처리하였을 때, 가스트린 promoter 의 agonist인 EGF를 처리하였을 때와 비슷한 정도로 유의적인 가스트린 promoter 활성의 증가를 나타내었다. 반면 EGF receptor kinase inhibitor인 AG1478을 처리하였을 때는 HB-EGF에 의해 유도된 가스트린 promoter 활성 증가가 유의적으로 감소되었다. 이러한 결과는 EGF receptor가 가스트린 promoter 활성을 유도하는데 있어 중요하게 작용한다는 것을 나타낸다. G240-Luc 세포주에 C-Raf siRNA를 처리하고 헬리코박터를 감염시키면 헬리코박터에 의해 유도된 가스트린 promoter 활성이 유의적으로 감소하였다. 반면 A-Raf siRNA 또는 B-Raf siRNA 처리는 가스트린 promoter 활성에 영향을 나타내지 않았다. 이러한 결과는 헬리코박터에 의해 유도되는 가스트린 promoter 활성조절에 C-Raf가 중요하게 관여한다는 것을 제시하는 것이다.

G240-Luc 세포주에 MEK1 siRNA를 처리하였을 때 헬리코박터 감염에 의해 유도된 가스트린 promoter 활성이 유의적으로 감소하였다. 하지만 MEK2 siRNA 처리는 가스트린 promoter 활성에 영향을 미치지 않았다. 이 결과를 통해 헬리코박터 감염에 의해 유도되는 가스트린 발현에 MEK1가 중요한 역할을 수행한다는 것을 알 수 있었다. 또한 MEK1과 MEK2 단백질이 구조적, 기능적으로 매우 유사함에도 불구하고 헬리코박터에 의해 유도되는 가스트린 발현에는 MEK1 가 주요하게 관여하는 것으로 판단된다.

ERK2 siRNA를 처리하였을 때 가스트린 promoter 활성은 유의적으로 감소하였고, ERK1 siRNA처리는 가스트린 promoter 활성을 유의적으로 증가시켰다. 이런 결과들은 ERK2는 헬리코박터 감염에 의한 가스트린 유도에 관여하며, ERK1는 가스트린 억제에 관여하는 것을 나타내며, ERK 단백질은 ERK heterodimer또는 ERK2 homodimer를 형성하여 잠재적인 가스트린 promoter activator로써 작용할 가능성이 있다는 것을 제시한다.

본 연구를 통하여 헬리코박터에 감염된 숙주 세포는 EGFR-C-Raf - MEK1-ERK2를 경유하는 신호전달 과정을 통하여 가스트린 발현을 유도한다는 것을 알 수 있었다. 또한, EGF 수용체를 통한 HB-EGF 신호전달 과정이 헬리코박터 감염에 의해 유도되는 가스트린 promoter 활성 과정에 매우 밀접하게 연관되어 것도 밝혀졌다. 흥미롭게도, MAPK 신호 전달 단백질 ERK1과 ERK2는 헬리코박터 감염에 의해 유도되는 가스트린 발현 과정에서 서로 상반되는 역할을 하고 있었다. 특히 ERK1의 가스트린 활성 억제능이 헬리코박터 감염에

의해 증가되는 가스트린 발현 조절에 중요한 역할을 한다고 생각된다. 이 연구는 헬리코박터 감염시 유도되는 가스트린 발현과 관련된 MAPK 신호전달 과정을 자세히 이해함으로써 헬리코박터에 의한 고가스트린 혈증 뿐만 아니라 위암유발을 감소시키기 위한 치료 전략 개발에 유용한 정보를 제공할 수 있을 것이다.

핵심 되는 말: 헬리코박터 파일로리, 가스트린, MAPK pathway, Raf, MEK, ERK1, ERK2