NOVEL EPIDERMAL GROWTH FACTOR RECEPTOR PATHWAY MEDIATES RELEASE OF HUMAN β-DEFENSIN 3 FROM *HELICOBACTER PYLORI*-INFECTED GASTRIC EPITHELIAL CELLS

ヘリコバクター・ピロリ感染胃上皮細胞からの
ヒト・ベータ・ディフェンシン３遊離に関わる新規 EGFR 経路の同定

By

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PREFACE

This thesis was submitted for the degree of Doctor of Philosophy (PhD) to the Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Japan. The results within this thesis were published in two peer-reviewed PubMed indexed medical journals. Details are shown below:

1. **Muhammad JS**, Zaidi SF, Zhou Y, Sakurai H, Sugiyama T. Novel epidermal growth factor receptor pathway mediates release of human β-defensin 3 from *Helicobacter pylori*-infected gastric epithelial cells. *Pathog Dis.* 2016; 74(3). pii: ftv128. doi: 10.1093/femspd/ftv128.
2. Zaidi SF*, Refaat A*, Zhou Y, **Muhammad JS**, Shin MS, Saiki I, Sakurai H, Sugiyama T. *Helicobacter pylori* Induces Serine Phosphorylation of EGFR via Novel TAK1-p38 Activation Pathway in an HB-EGF-Independent Manner. *Helicobacter*. 2015; 20(5):381-9.

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ABSTRACT

***Background*:** Persistent *Helicobacter pylori* (*H. pylori*) infection induces to express anti-microbial peptides from gastric epithelial cells, especially human β-defensin 3 (hBD3), as an innate immune response, and this expression of hBD3 is mediated by epidermal growth factor receptor (EGFR) activation. In this study, we have demonstrated the role of *H. pylori* in the activation of EGFR via transforming growth factor β-activated kinase-1 (TAK1)-mediated phosphorylation of p38α. We also found that phosphorylation of a serine residue of EGFR via TAK1 and subsequent p38α activation is essential for *H. pylori*-induced hBD3 release from gastric epithelial cells. We showed that this pathway was dependent on *H. pylori* type IV secretion system (T4SS) and was independent of *H. pylori*-derived CagA or peptidoglycan.

***Materials and methods*:** Gastric epithelial (AGS or MKN-45) cells were co-cultured with wild type *H. pylori*, △*vir*B4 (T4SS mutant) *H. pylori* or cagA^{-} *H. pylori*. *H. pylori* was added to the cells and the activation of hBDs, EGFR, p38α, and TAK1 were examined by real-time reverse transcription polymerase chain reactions (RT-PCR) and western blotting. Infected cells were pretreated with or without EGFR ligands, chemical inhibitors, anti-HB-EGF antibody, and/or siRNAs to evaluate the effects of p38α, TAK1, NOD1 and EGFR on the release of hBD3. Fluorescence microscopy and flow cytometry were performed to detect the internalization of EGFR.

***Results*:** *H. pylori* infection induced phosphorylation of serine residue of EGFR, and this phosphorylation was followed by internalization of EGFR; consequently hBD3 was released at an early phase of the infection. Incubating cells with wild

type and CagA^{-} *H. pylori* strains resulted in the rapid and transient phosphorylation of serine residues of EGFR. RNAi experiments using siRNA against TAK1 and p38α pathways blocked the phosphorylation of serine residue. Immunofluorescence and flow cytometry revealed that EGFR was internalized in *H. pylori*-infected cells after EGFR phosphorylation in a p38α-dependent manner. In contrast, pre-treatment with anti-HB-EGF antibody did not block both the phosphorylation and internalization of EGFR. In the presence of TAK1 or p38α inhibitors, synthesis of hBD3 was completely inhibited. Similar results were observed in EGFR-, TAK1- or p38α-knockdown cells. However, NOD1 knockdown in gastric epithelial cells did not inhibit hBD3 induction.

***Conclusion*:** *H. pylori* induces internalization of EGFR via novel TAK1-p38α activation pathway which is independent of HB-EGF. Further, our study has firstly demonstrated that this novel EGFR activating pathway functioned to induce hBD3 at an early phase of *H. pylori* infection. This study provides an understanding of how *H. pylori* survive and persists in the hostile gastric mucosa; this along with other similar studies might help in the development of effective strategies to overcome *H. pylori* infection.

KEYWORDS

1. *Helicobacter pylori;*
2. Gastric epithelial cells;
3. Host immune response;
4. Anti-microbial peptides;
5. Human β-defensin-3;
6. Epidermal growth factor receptor;
7. Heparin binding-epidermal growth factor;
8. Transactivation pathway
9. p38α;
10. Transforming growth factor β-activated kinase-1;

CHAPTER 1

1. BACKGROUND

The gram-negative bacterium *Helicobacter pylori (H. pylori)* persistently colonizes the human stomach and also have been the focus of basic biochemical and clinical research since it was first identified and cultured by Marshall and Warren in 1982 (Warren and Marshall, 1983). The prevalence rate of *H. pylori* and its associated diseases has been highly inconsistent worldwide. In industrialized countries there is generally a low prevalence of *H. pylori* infection and yet a relatively high prevalence of gastric cancer. On the other hand, some countries with high *H. pylori* prevalence rates have low gastric cancer prevalence particularly among the Asian countries. Prevalence of *H. pylori* infection is high in developing Asian countries like India, Bangladesh, Pakistan, and Thailand and is acquired at an early age compared to developed Asian countries like Japan and China. However, the frequency of gastric cancer is very low in India, Bangladesh, Pakistan and Thailand than that of Japan and China. Similar enigma has been reported from Africa as compared to the West (Graham, Lu and Yamaoka, 2009).

H. pylori is highly adapted to the gastric environment where it lives within or beneath the gastric mucous layer. *H. pylori* render the underlying gastric mucosa more vulnerable to acid peptic damage by disrupting the mucous layer, liberating enzymes and toxins, and adhering to the gastric epithelium (Muhammad, Zaidi and Sugiyama, 2012). In spite the induction of strong host inflammatory immune responses, *H. pylori* persist in a hostile gastric environment in nearly half of the world's human population (Blaser and Atherton, 2004). Some infected individuals will develop gastric ulcer, duodenal ulcer,

gastric carcinoma, or gastric mucosa-associated lymphoid tissue lymphoma; however, the majority of infected individuals remain asymptomatic. Disease diversity following *H. pylori* infection depends mainly on bacterial virulence factors, host immune responses, environmental factors, or combination of them (Muhammad, Sugiyama and Zaidi, 2013).

Virulent strains of *H. pylori* encode *cag pathogenicity island* (*cag*PAI), which expresses a type IV secretion system (T4SS). This T4SS forms a syringe-like pilus structure for the injection of virulence factors such as the CagA effector protein into host target cells (*See figure 1.1, page 10*). This is achieved by a number of T4SS proteins, including CagI, CagL, CagY and CagA, which by itself binds to the host cell integrin member β-1 followed by delivery of CagA across the host cell membrane. A role of CagA interaction with phosphatidylserine has also been shown to be important for the injection process. After delivery, CagA becomes phosphorylated by oncogenic tyrosine kinases (e.g., Src Kinase) and mimics a host cell factor for the activation or inactivation of some specific intracellular signaling pathways i.e. protein tyrosine phosphatase pathway (Jenks and Kusters, 2000; Higashi *et al*., 2002; Naumann, 2005; Tegtmeyer, Wessler and Backert, 2011; Posselt, Backert and Wessler, 2013). The presence of a functional *cag*PAI segment integrated in *H. pylori* is associated with more severe gastritis and augments the risk of developing gastric cancer (Peek, 2002). The knocking out of this T4SS deprived wild-type *H. pylori* of its pathogenicity for causing gastritis and gastric ulcer in a gerbil model, thereby highlighting the critical role of T4SS encoded by the *cag*PAI (Ogura *et al*., 2000). Approximately 85-100% of patients with duodenal ulcers have $CagA^+$

strains, compared to 30-60% of infected patients who do not develop ulcers (Fallone et al., 2000). Similarly, *cagA*$^{+}$ *H. pylori* strains compared with *cagA*$^{-}$ strains significantly increased the risk of developing severe gastritis and gastric carcinoma (Blaser *et al*., 1995).

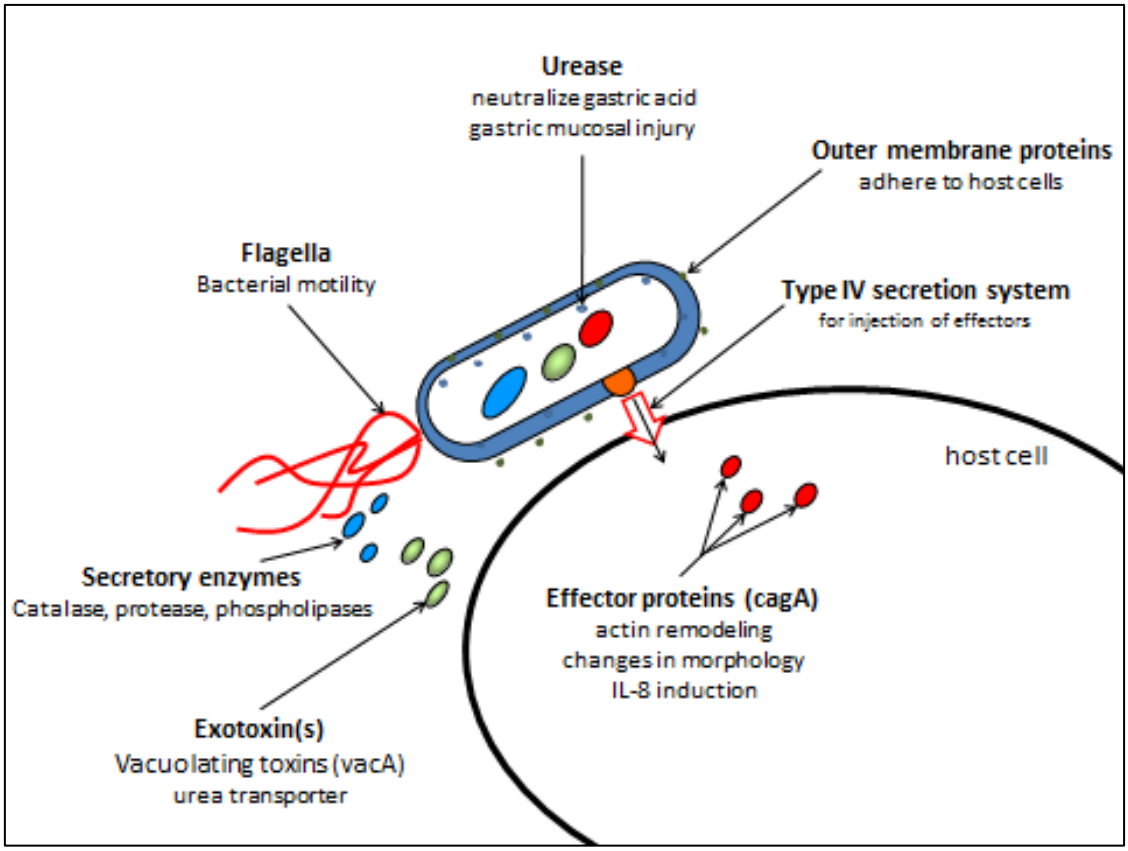

Figure 1: Bacterial factors responsible for virulence of *H. pylori***.**
(Muhammad *et. al., J Pak Med Assoc* (2013) 63:1528-33)

H. pylori produces a number of antigenic substances which can be taken up and processed by macrophages in the lamina propria of gastric mucosa and then activate T-cells to facilitate immune stimulation (Portal-Celhay and Perez-Perez, 2006). This *H. pylori* interaction with gastric epithelial cells results in production of inflammatory cytokines like interleukin-8, activation of

transcription factors like nuclear factor kappa B (NF-κB), and disturbances in cell proliferation and morphology (Hatakeyama and Higashi, 2005; Keates *et al*., 1997). NF-κB has an essential role in the regulation of inflammatory responses in mammals. The prototypical NF-κB complex, which is a heterodimer of p50 and RelA, is sequestered in the cytoplasm by its inhibitor IκBα. On stimulation, the IκB kinase (IKK) complex is activated, leading to phosphorylation and degradation of IκBα, nuclear translocation of NF-κB and activation of its target genes (Gosh and Karin, 2002). Transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) is a key regulator of signal transduction cascades that lead to stimulus-coupled phosphorylation and activation of IKK (Sakurai, 2012). Previous studies also showed that TAK1 is polyubiquitinated by TRAF6 in response to TGF-β, and that Lys 63-linked ubiquitination is required for TGF-β-induced activation of p38α/Jun N-terminal kinase and AP-1, indicating that TAK1 ubiquitination might also be crucial for the activation of IKK and NF-κB (Sorrentino *et al*., 2008). Role of CagA protein in activation of TAK1 and NF-κB is controversial. Lamb *et al* demonstrated the role of CagA in direct activation of TAK1 through the TRAF6-mediated, Lys 63-linked ubiquitination of TAK1 in gastric cells (Lamb *et al*., 2009). In contrast, a recent report by Sokolova *et al* documented a type IV secretion system-dependent and CagA-independent activation of TAK1 and NF-κB in AGS cells (Sokolova *et al*., 2014). Therefore, the role of CagA in the activation of TAK1 is controversial and still unclear.

CHAPTER 2

2. INTRODUCTION

Gastric epithelial cells are known to release various anti-microbial peptides (AMPs) upon *H. pylori* infection. Human β-defensins (hBDs) are the most potent AMPs released by gastric epithelial cells in response to persistent *H. pylori* infection (George *et al*., 2003). These short cationic, disulfide-linked peptides possess strong antimicrobial activity against many Gram-positive and Gram-negative bacteria (Guaní-Guerra *et al*., 2010). The first three members of hBD family (hBD1-3) have been extensively investigated as important host defense factors against *H. pylori* infection (George *et al*., 2003; Bajaj-Elliot *et al*., 2002; Boughan *et al*., 2006; Grubman *et al*., 2010; Bauer *et al*., 2013). Interestingly, hBD1 is expressed in uninfected gastric epithelial cells (O'Neil *et al*., 2000), and a recent study showed that hBD1 was downregulated in gastric epithelial cells upon *H. pylori* infection (Patel *et al*., 2013; Taha *et al*., 2005). Notably, hBD1 has a weak anti-*H. pylori* activity in comparison to hBD2 and hBD3 based on *in vitro* findings (Nuding *et al*., 2013). Regarding hBD2 and hBD3 anti-bacterial activity, hBD3 can exert 100 times more potent anti-*H. pylori* activity than hBD2 (Bauer *et al*., 2012; Kawauchi *et al*., 2006). Therefore, hBD3 release from *H. pylori*-infected gastric epithelial cells can be considered as an essential, first-line host immune response. The expression of hBD3 is reportedly dependent on the EGFR/MAP kinase pathway (Boughan *et al*., 2006) *(see figure 2.1, page 14)*. However, the molecular pathway that drives hBD3 induction in *H. pylori*-infected cells has not been fully determined.

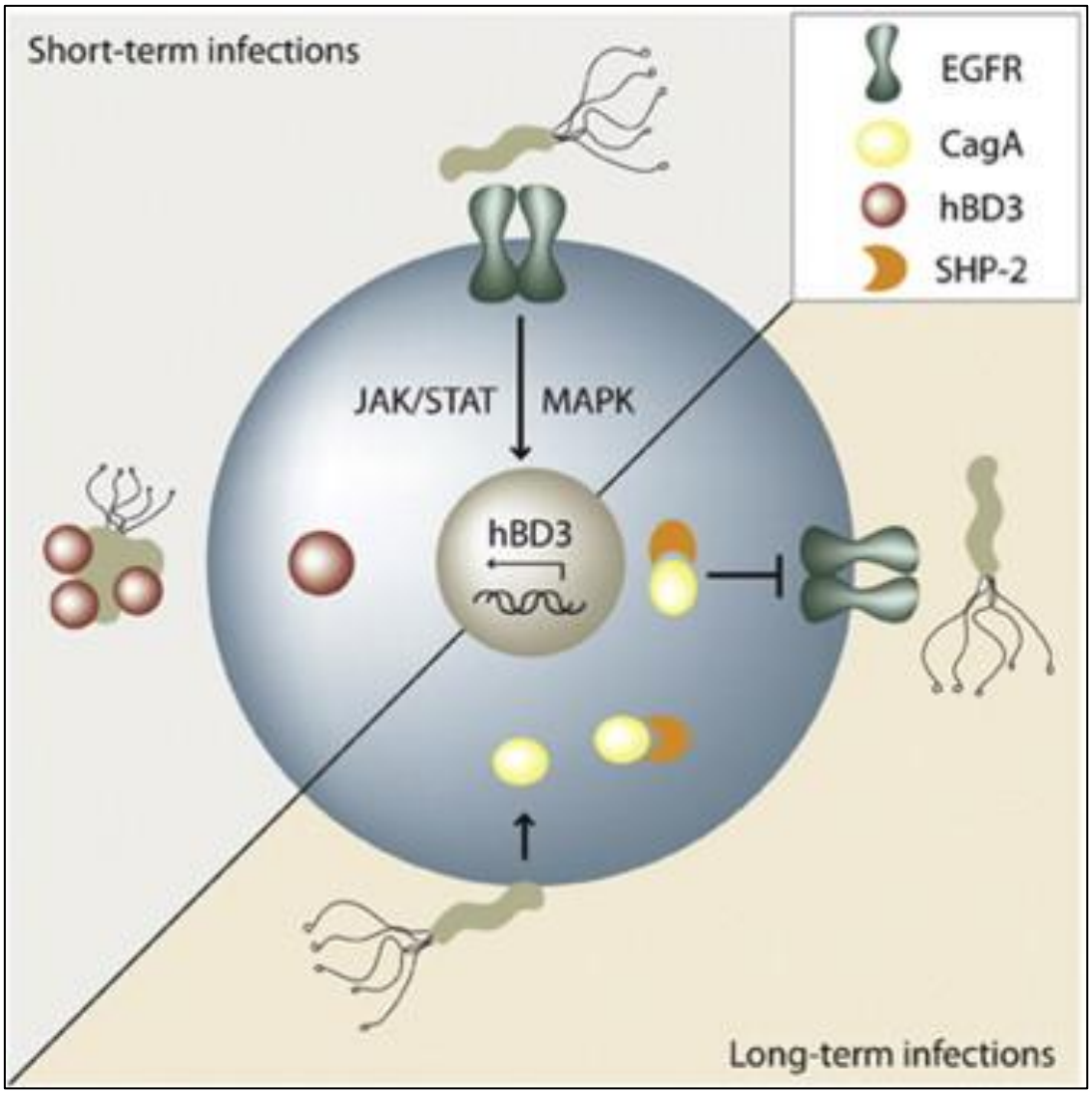

Figure 2.1: Release of hBD3 from gastric epithelial cells
(Bauer *et. al., Cell Host Microbe* (2012) **11**:576-86)

Overexpression and transactivation of epidermal growth factor (EGF) receptor (EGFR) by *H*. *pylori* has been reported earlier (Ashktorab *et al*., 2007; Keates *et al*., 2001; Keates *et al*., 2007). EGFR is a member of the ErbB family, which consists of four tyrosine kinase receptors: EGFR (ErbB1) and ErbB2–4. These four receptors plays a critical role in a wide variety of cellular functions, including proliferation, differentiation, and apoptosis (Citri and Yarden, 2006; Schneider and Wolf, 2009). EGFR has recently been a focus of molecular targeted cancer therapy, because overexpression, amplification, and mutations are involved in carcinogenesis and the progression of several types of cancer (Mendelsohn and Baselga, 2003).

H. *pylori* was known to induce the activation of EGFR by increasing the expression and release of heparin binding-epidermal growth factor (HB-EGF) from gastric epithelial cells, a process termed as "transactivation" (Dickson *et al*., 2006) *(see figure 2.2, page 16)*. HB-EGF, a member of EGF family, is synthesized by macrophages and cultured cells as a membrane-anchored propeptide which is proteolytically processed to release a mature soluble factor (Higashiyama *et al*., 1991). Recently, a study reported an intracellular pathway the involving TAK1 in EGFR phosphorylation and subsequent endocytosis via mitogen-activated protein kinases (MAPK) in TNF-α stimulated HeLa cells (Nishimura *et al*., 2009). Nevertheless, the functional significance of this novel EGFR activation pathway was never analyzed in *H. pylori*-infected gastric epithelial cells *(see figure 2.3, page 17)*.

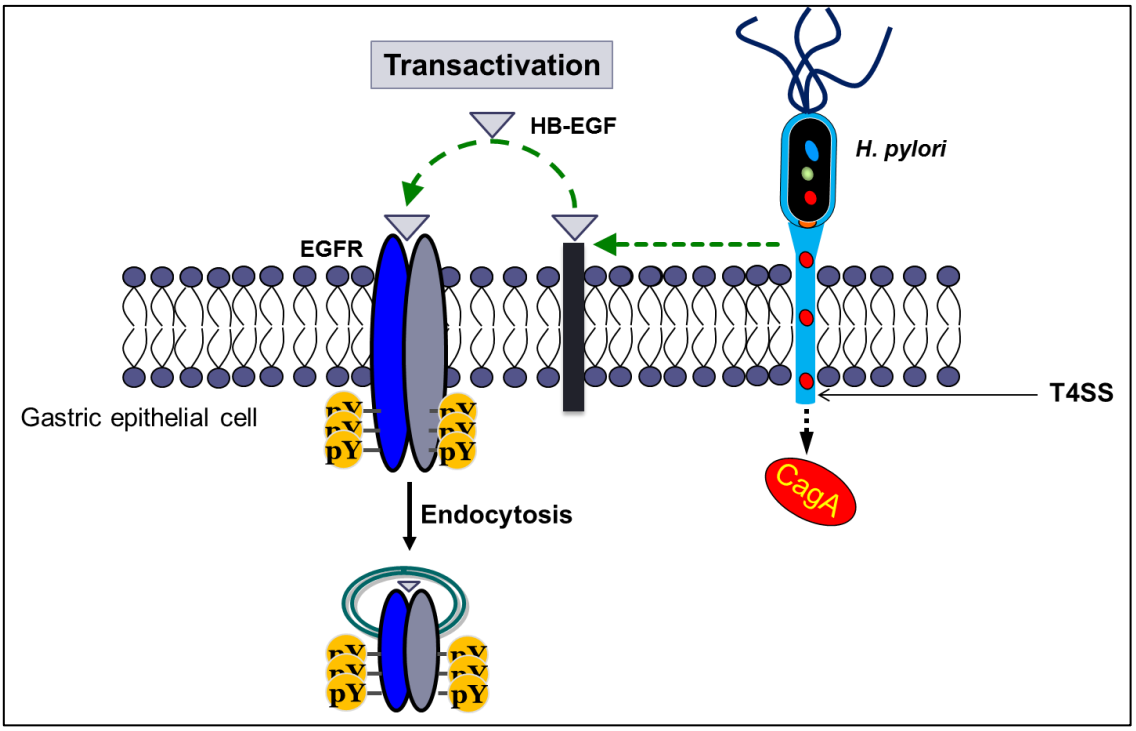

Figure 2.2: Transactivation of EGFR by *H. pylori* in gastric epithelial cells (Keates *et. al.*, J Biol Chem (2001) 276: 48127-34)

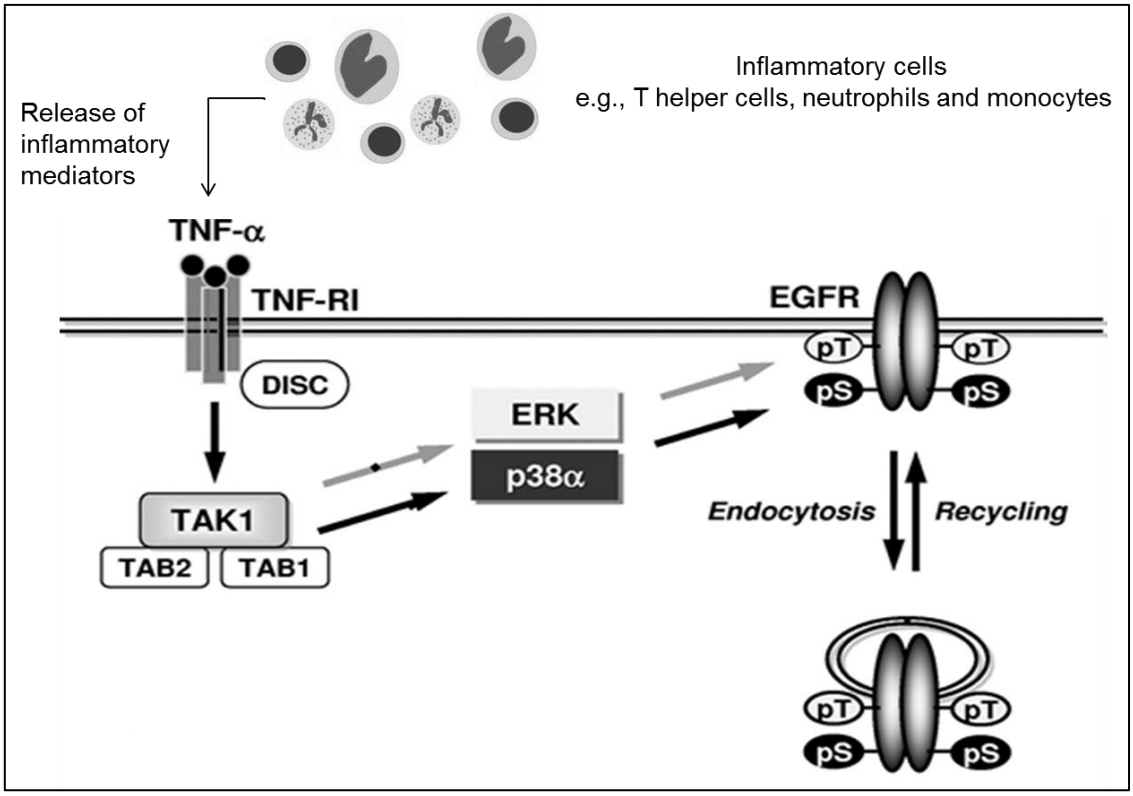

Figure 2.3: Activation of TAK1 and EGFR by TNF-α in non-gastric cells (Nishimura *et. al.,* Molecular and cellular biology (2009) 29:5529-39)

CHAPTER 3

3. AIMS OF THE STUDY

The aim of this study was to demonstrate that *H*. *pylori* infection of gastric epithelial cells causes the phosphorylation of EGFR serine residue via TAK1-p38α pathway, and that the EGFR serine residue phosphorylation is essential and specific in endocytosis of EGFR in an HB-EGF independent manner.

Also this study was aimed to show that EGFR activation via phosphorylation of a specific serine residue of EGFR via a TAK1-p38α pathway and the subsequent internalization of EGFR are functionally linked to the induction of hBD3 in gastric epithelial cells infected with *H. pylori*.

CHAPTER 4

4. MATERIALS AND METHODS

4.1 Bacterial strains and culture conditions

H. pylori strain 193C originated from a patient with gastric cancer, and the isogenic T4SS mutant *H. pylori* (△*vir*B4) was developed from *H. pylori* 193C by T. Sugiyama (Mizushima *et al*., 2002). Strain CPY2052 was isolated at Yamaguchi University Hospital, Japan, from a patient with a gastric ulcer (Tsuda, Karita and Nakazawa, 1993), and *H. pylori* knocked-out mutants (CP1 CagA¯) were developed from a CPY2052 strain by Dr. J. Akada, Yamaguchi University, Japan (Akada *et al*., 2010). *H. pylori* were cultured in Brucella broth medium (BB) supplemented with 10% fetal bovine serum (FBS) under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2 at 37ºC; Sanyo-Multigas Incubator, SANYO Electric Co., Ltd. Tokyo, Japan) with 100% humidity on a gyratory shaker set at 160 rpm. The formula absorbance of 0.1 = 10^8 bacteria/ml was used to estimate the concentration of bacteria in each culture.

4.2 Cell culture and co-culture conditions

The human gastric carcinoma cell lines AGS and MKN-45 were purchased from American Type Culture Collection (Manassas, VA, USA); cells were grown in RPMI 1640 containing 2 mmol/L L-glutamine and 10% FBS at 37ºC in 5% CO_2. The cells were routinely passaged every 3 days. Each cell line was authenticated by National Institute of Biomedical Innovation, Osaka, Japan as identical to the corresponding cell line registered in the Japanese Collection of Research Bio-resources Cell Bank (JCRB) database. Cells were seeded into 6cm culture dish and grown for 24 h; these cultures were then washed with phosphate-buffered saline (PBS) three times before co-culture. Fresh RPMI

1640 medium without antibiotics or FBS was added 1 h before addition of *H. pylori*. Before each co-culture experiment, *H. pylori* were cultured overnight in BB-FBS-10% under the conditions described above and then washed twice with PBS; these bacteria were then directly added to AGS/MKN-45 cells for the indicated times.

4.3 Stimulants, antibodies and inhibitors

A selective TAK1 inhibitor (5Z-7-oxozeaenol (5OZ)) (Ninomiya-Tsuji *et al*., 2003), TNF-α, HB-EGF, and human EGF were purchased from R&D systems (Minneapolis, MN, USA). Antibodies against NOD1, p38α, phospho-p38α (Thr-180/Tyr-182) or EGFR (Ser-1046/7, Tyr-992, Tyr-1045, Tyr-1068) were purchased from Cell Signaling Technology (Danver, MA, USA). Antibodies against TAK1 (M-579), phospho-TAK1, total EGFR (1005), hBD3 (FL-67), or Actin (C-11) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Mouse anti-*H. pylori* CagA (B237H) antibody was purchased from Abcam Biotechnologies (Cambridge, UK), and anti-*H. pylori* UreA antibody was purchased from Dako (Carpinteria, CA, USA). SB203580 (SB), a chemical inhibitor of p38α, was purchased from Merck Biosciences (Whitehouse Station, NJ, USA). The SB and 5OZ were each dissolved separately in dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries Ltd, Osaka, Japan), and the final concentration of DMSO was less than 0.1%.

4.4 Quantitative reverse transcription polymerase chain reaction (RT-PCR)

In order to evaluate hBD1, hBD2, and hBD3 expression, cells were co-cultured with *H. pylori* for 1 h to 48 h, then washed with PBS three times, and then scrapped from the dish substratum with a rubber policeman (Thermo Fischer

Scientific Inc., Waltham, MA, USA); RNeasy Plus Mini kits (Qiagen, Hilden, Germany) were then used according to the manufacturer's instructions to extract total RNA from cells. Exscript RT Reagent Kits (Takara Bio Inc., Shiga, Japan) and random primers were used for reverse transcription of 1 μg of total RNA; RT-qPCR was then performed as follows: PCR amplification of mRNA encoding each hBD (1, 2, or 3) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed using SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan). Quantification of hBD1 mRNA was carried out using commercial primers (Qiagen, Hilden, Germany); for hBD2 mRNA, the forward 5′-CTGATGCCTCTTCCAGGTGTTT-3′ and reverse 5'-GAGACCACAGGTGCCAATTTG-3' primers were used; for hBD3 mRNA, the forward 5'-TCTGCCTTACCATTGGGTTC-3' and reverse 5'-CACGCTGAGACTGGATGAAA-3' primers were used; and for GAPDH, the forward 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse 5'-TGGTGAAGACGCCAGTGGA-3' primers were used. The Mx3000 QPCR system (Agilent Tech., Santa Clara, CA, USA) was used for RT-qPCR. The PCR cycling conditions were as follows: an initial denaturation step for 10 min at 95°C; then 40 cycles at 95°C (30 s), 60°C (30 s) and 72°C (30 s); 3 min at 95°C; and finally 10 s at 50°C. Expression of each hBD mRNA was normalized relative to GAPDH mRNA expression, which functioned as an internal control in each sample. The hBD expression in a sample was determined by comparing hBD mRNA amplification with those of standard PCR products of known concentration. The standards of known concentrations were prepared by purifying the PCR products of all hBDs genes in *H. pylori* infected samples using

the PCR clean-up system (Promega, Madison, WI, USA). Relative expression percentages were calculated as the percentage of an individual hBD mRNA relative to GAPDH mRNA (hBD/GAPDH*100); the average of three independent experiments, each with PCR reaction efficiencies of more than 80%, were used to calculate relative expression

4.5 Western blotting

AGS cells were pretreated with the indicated specific inhibitor or stimulant for 30 min and then were exposed to *H. pylori*. After the co-culture experiment with *H. pylori*, gastric epithelial cells were rigorously washed with ice-cold PBS six times to ensure removal of adherent extracellular *H. pylori*. Whole-cell lysates were prepared from AGS or MKN-45 cells with whole-cell lysis buffer as described previously (Zaidi *et al*., 2009). However, to prepare bacterial cell lysates for western blotting analysis, a special Bacterial Protein Extraction Reagent (B-PER) (Thermo Scientific, Rockford, IL, USA) was used instead of whole-cell lysis buffer, which failed to extract *H. pylori* protein. Proteins in cell lysates were then resolved by SDS-PAGE and transferred to an Immobilon-P nylon membrane (Merck Millipore, Billerica, MA, USA). Each membrane was treated with BlockAce (Dainippon Pharmaceutical Co., Ltd., Suita, Japan) and probed with the indicated primary antibodies. Primary antibodies were detected with horseradish peroxidase-conjugated anti-rabbit, anti-mouse, or anti-goat immunoglobulin G (Dako) and then visualized with ImageQuant LAS4000 (GE Healthcare, Little Chalfont, Buckinghamshire, UK). To enhance the immunoreaction, some antibody reactions were conducted in Can Get Signal solution (Toyobo corp., Osaka, Japan).

4.6 Flow cytometry

After the stimulation of AGS cells with *H*. *pylori*, HB-EGF or αHB-EGF, those cells were harvested in PBS. Those treated AGS cells were fixed with 2% paraformaldehyde for 20 min at room temperature. Cells were re-suspended in 100 μl of FACS buffer (PBS containing 0.5% bovine serum albumin and 0.05% NaN_3) containing 1 μg of anti-EGFR monoclonal antibody (clone LA1; Upsate) and then were incubated on ice for 30 min. After that, the cells were washed with FACS buffer; next the cells were incubated with fluorescein isothiocynate-conjugated anti-mouse IgG antibody (Dako) on ice for 30 min and analyzed by the FACSCalibur system (BD). Experiments were carried out minimally two times and the representative results were shown.

4.7 RNA interference

Small interfering RNAs (siRNAs) were synthesized at Hokkaido System Science Co., Ltd. (Hokkaido, Japan). Target sequences against NOD1, EGFR, TAK1, p38α, and firefly luciferase genes were:

5'-GGCCAAAGUCUAUGAAGAUTT-3',

5'-CCUAUGCCUUAGCAGUCUUAUCUAA-3',

5'-UGGCUUAUCUUACACUGGA-3',

5'-GCAUUACAACCAGACAGUUGAUAUU-3', and

5'-CGUACGCGGAAUACUUCGA-3', respectively.

Lipofectamine 2000 (Life Tech., Carlsbad, CA, USA) was used to transfect siRNAs in a final concentration of 20 nM into AGS cells. Approximately 72 h after transfection, cells were co-incubated with *H. pylori* for 60 min or 8 h.

4.8 Immunofluorescence microscopy

To monitor EGFR internalization, AGS cells were seeded onto individual glass-bottom culture dishes (FastGene, Nippon Genetics, Tokyo, Japan) and co-cultured with *H. pylori* for 60 or 120 min. To evaluate effect of siTAK or sip38α on EGFR internalization, Lipofectamine 2000 (Life Tech., Carlsbad, CA, USA) was used to transfect AGS cells with an individual siRNA at a final concentration of 20 nM. Approximately 72 h after transfection, *H. pylori* were added to AGS cells and the co-cultures were incubated for 60 min. Cells were fixed with paraformaldehyde, subsequently stained with anti-EGFR antibody clone LA1 (Merck Millipore, Billerica, MA, USA), and then with Alexa-488-conjugated anti-mouse IgG antibody (Life Tech., Carlsbad, CA, USA); immunofluorescence was analyzed with a LSM 700 confocal microscope (Zeiss, Jena, Germany). Three independent experiments were performed.

4.9 Statistical analysis

The student's t-test was used to evaluate the significance of differences between groups; Microsoft Excel 2013 was used for all statistical calculations. At least three independent replicates were used for each experiment, and pooled data are represented as mean ± SEM. The level of significance was set at $p < 0.01$. Mean and standard deviation were used to assess quantitative measures for RT-PCR results. For western blotting, flow cytometry, and immunofluorescence one representative figure out of three independent experiments is shown.

CHAPTER 5

5. RESULTS

5.1 Phosphorylation of TAK1, p38α, and residues of EGFR by TNF-α and H. pylori *stimulation in gastric epithelial cells*

As reported earlier, TNF-α induces phosphorylation of TAK1, p38α, serine and threonine residues of EGFR in non-gastric (HeLa) cells (Nishimura *et al*., 2009). In this study, we first confirmed the similar effect in gastric epithelial cells. TNF-α triggered phosphorylation of serine and threonine residues of EGFR after 5 min of incubation without any effect on tyrosine residue (Fig 5.1A). Next, we evaluated the effect of *H. pylori* in AGS cells on phosphorylation of TAK1, and p38α. *H. pylori* induced the phosphorylation of TAK1, and p38α in a time-dependent manner. TAK1 activation was highest at 30 min of incubation while phosphorylation of p38α was peaked at 45 min (Fig 5.1B). We examined phosphorylation of serine (pS1046/7) and threonine (pT669) residues of EGFR in *H. pylori*-infected cells. Serine and threonine residues of EGFR were phosphorylated after 30 min of *H. pylori* co-culture and decreased at 120 min (Fig. 5.1C). We compared the effect EGF, HB-EGF, *H. pylori* and TNF-α stimulation on serine and threonine phosphorylation of EGFR at mentioned time intervals. All four stimulatory agents induced phosphorylation of threonine residue while only *H. pylori* and TNF-α brought about phosphorylation in serine residue (Fig 5.1D). This shows that serine phosphorylation of EGFR is quite specific with *H. pylori* infection in AGS cells and not related to other ligands of EGFR.

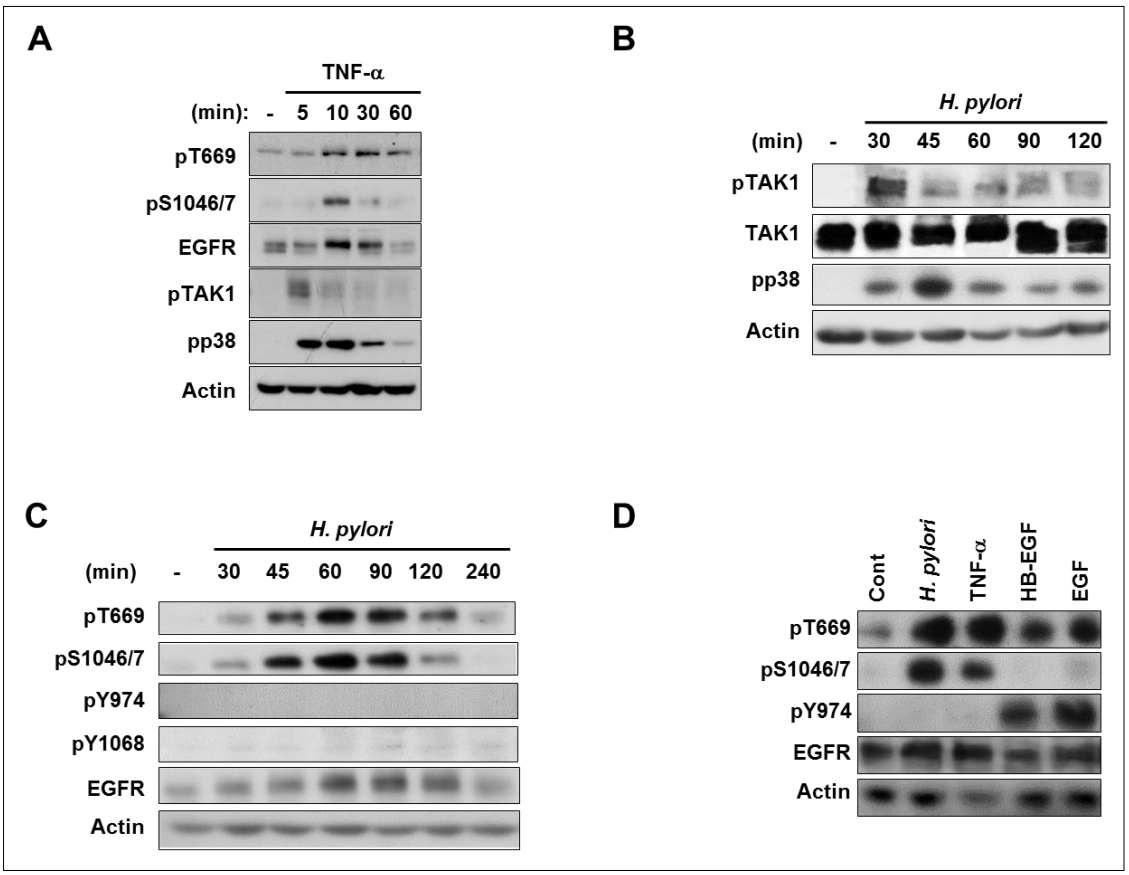

Figure 5.1: Phosphorylation of residues of EGFR, activation of TAK1, p38α and EGFR in TNF-α stimulated and *H. pylori*-infected gastric epithelial cells.

A, AGS cells treated with 10 ng/ml TNF-α for 5, 10, 30, or 60 min; **B**, Effect of *H. pylori* on activation of TAK1 and p38α; **C**, Phosphorylation of serine and threonine residues of EGFR by *H. pylori*. D, A comparative analysis of phosphorylation of serine/threonine, and tyrosine residues of EGFR by *H. pylori*, TNF-α, HB-EGF, and EGF. AGS cells were incubated with *H. pylori* (60 min), TNF-α (10 min), HB-EGF (10 min), and EGF (10 min) with subsequent lysis and immunoblot analysis with phospho-EGFR, total EGFR, and Actin antibodies.
Each western blot shown is representative of three independent experiments. Multiplicity of *H. pylori* infection was 50:1.

5.2 Internalization of EGFR by H. pylori *in gastric epithelial cells*

Endocytosis of EGFR was analyzed by fluorescence microscopy (Fig. 5.2A). EGFR was drastically internalized after 60 min of *H. pylori* inoculation. We further confirmed the internalization of EGFR by flow cytometry after *H. pylori* co-culture at different point intervals. Maximum internalization of EGFR was evident at 60 min of *H. pylori* co-culture (Fig. 5.2B). This pattern of internalization correlated with our microscopy results. These results clearly demonstrated that *H. pylori* induces internalization of EGFR.

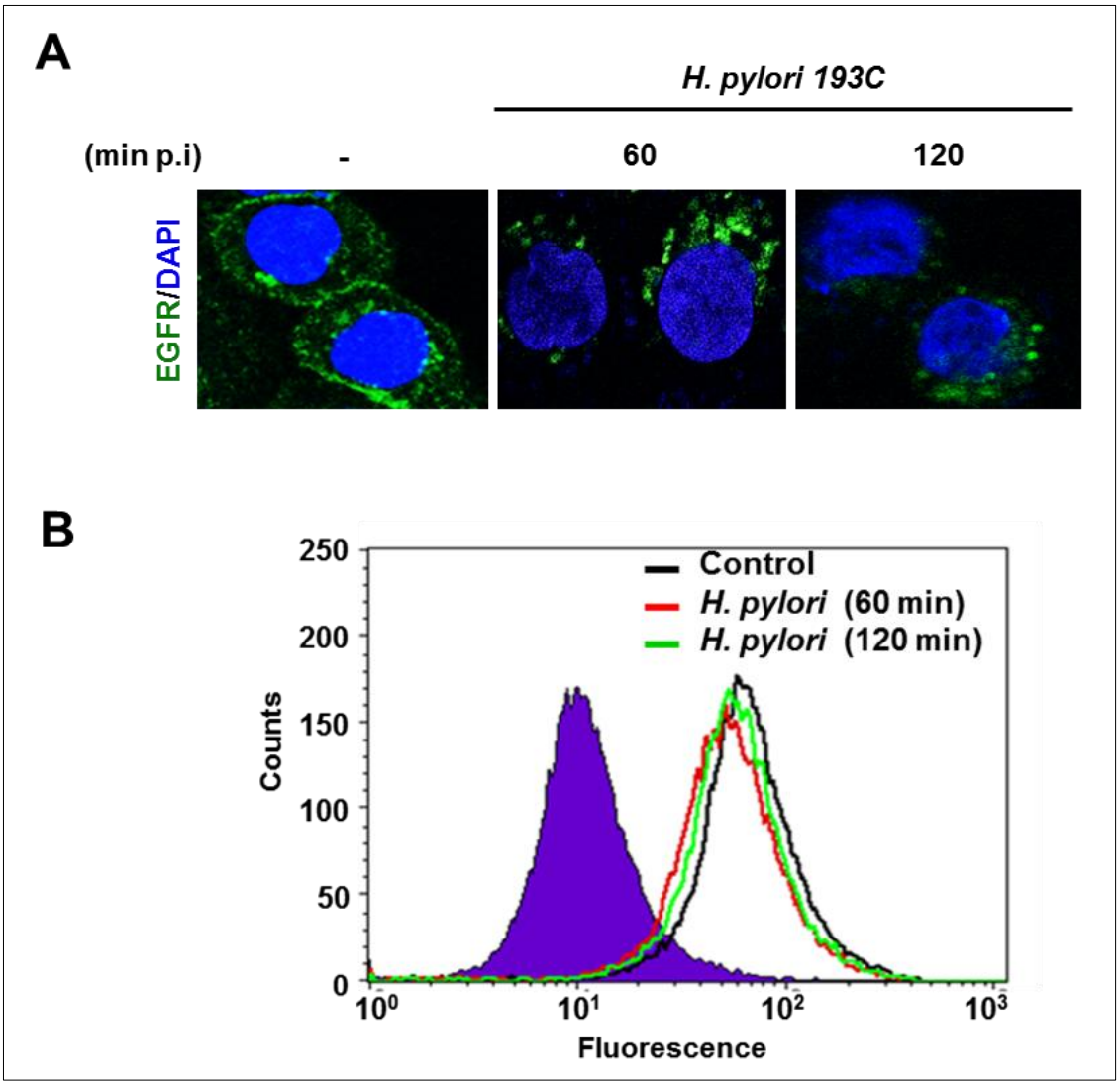

Figure 5.2: Internalization of EGFR by *H. pylori* in gastric epithelial cells.

A and **B**, AGS cells were incubated with *H. pylori* for 60 and 120 min and subcellular localization of EGFR was examined by confocal fluorescent microscopy (A) or cell surface expression of EGFR was investigated by flow cytometric analysis (B).

Multiplicity of *H. pylori* infection was 50:1. Each figure shown is representative of three independent experiments.

5.3 Effect of CagA¯ and △virB4 H. pylori *strains on phosphorylation of serine residues of EGFR*

AGS cells infected with *H. pylori* 193C or CPY2052 showed specific phosphorylation of a serine residue of EGFR (pS1046/7); however, tyrosine residue (pY1068) was not phosphorylated (Fig. 5.3A). Time-course analysis showed pS1046/7 phosphorylation levels peaked 60-120 min after initial co-culture with *H. pylori*. A similar serine phosphorylation was observed in AGS cells infected with CagA knockout *H. pylori* CP1; and CagA protein was not expressed in the host cells infected with *H. pylori* CP1 (Fig. 5.3B). To address the involvement of cagPAI and T4SS in inducing phosphorylation of serine residues of EGFR, we employed a △*virB4* (impaired T4SS) *H. pylori* strain and co-cultured it with the gastric epithelial cell lines, AGS. The △virB4 *H. pylori* failed to induce phosphorylation of serine at indicated time intervals compared to wild-type strain in both cell lines (Fig 5.3C).

To analyze the presence of CagA in each *H. pylori* strains bacterial lysates were prepared using B-PER bacteria lysis buffer. CagA expression in each *H. pylori* strain is shown in figure 5.3D. We performed genome sequencing of 3' region of CagA for both the wild-type strain of *H. pylori* strains (193C and CPY2052) used in this study. As both the strains were derived from Japanese patients, the 3' region of CagA of each strain showed similar EPIYA-ABD motif sequence *(see supplementary figure 1, page 68)*. Hence, the major pathogenic region of CagA of both the *H. pylori* strain is similar.

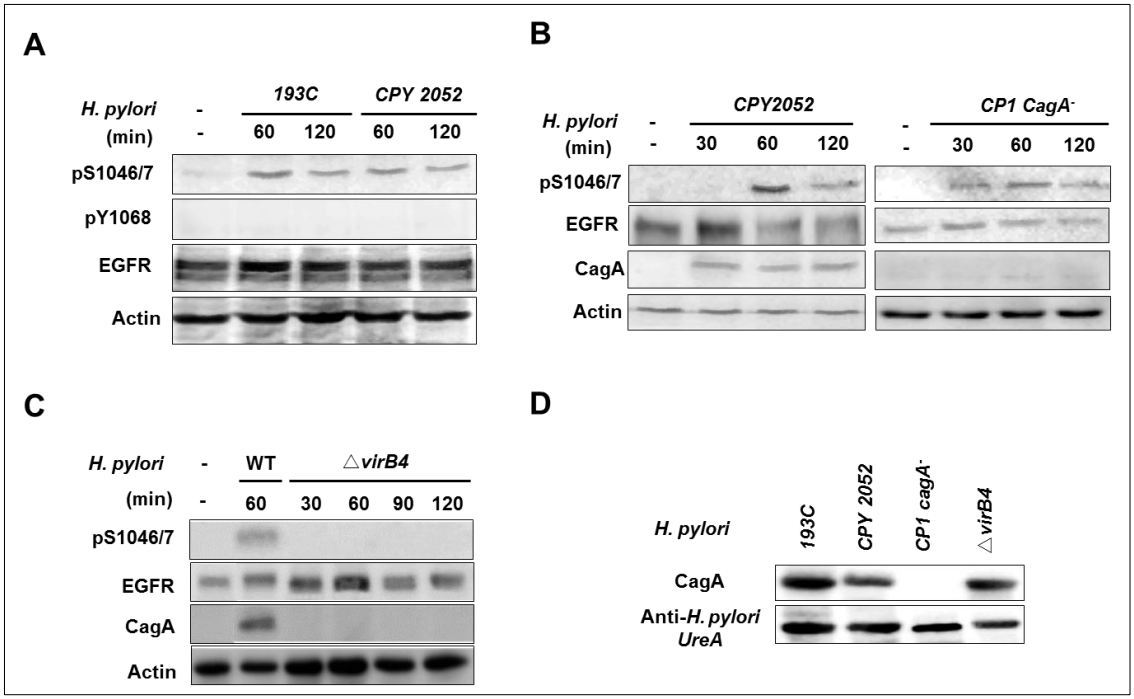

Figure 5.3: Effect of wild-type (WT), CagA¯ and △*vir*B4 *H. pylori* strains on phosphorylation of serine residues of EGFR.

A and **B**, AGS cells were infected with *H. pylori* 193C, CPY2052, or CP1 (CagA¯) for 60 or 120 min. Phosphorylation of serine residue of EGFR was evaluated by western blot. Each *H. pylori* strain strongly induced serine phosphorylation during early-phase infection. Activation of EGFR can also be noted as slight upwards shift of the EGFR band 60 and 120 min post infection; **C**, Wild type *H. pylori* (193C) and type IV secretion machinery deleted (△*vir*B4) strains were cultured in Brucella broth while AGS cells were cultured in RPMI1640 and subsequently co-incubated as mentioned before for indicated time intervals; **D**, Bacterial protein was extracted using B-PER from each *H. pylori* strain to evaluate presence of CagA protein using anti-CagA antibody. Anti-*H. pylori* UreA antibody was used as an internal control.

Each western blot shown is representative of three independent experiments. Multiplicity of *H. pylori* infection was 50:1.

5.4 Role of HB-EGF in phosphorylation of serine residue and EGFR endocytosis

To rule out the involvement of HB-EGF in phosphorylation and endocytosis of EGFR, we employed anti-HB-EGF neutralizing antibody in *H. pylori*-co-cultured cells. EGFR transactivation implicates triple membrane passing signal (TMPS) transmission events which involves activation of G-protein-coupled receptors, induction of extracellular transmembrane metalloprotease cleavage of pro-HB-EGF, and EGFR signaling by HB-EGF in an autocrine or paracrine manner (Prenzel *et al*., 1999). *H. pylori* has been documented to have matrix metalloproteinase activity, raising the possibility of direct bacterial cleavage, leading to release of HB-EGF (Gooz M, Gooz P, and Smolka, 2001). In addition, members of ADAM family have also been regulated by *H. pylori* infection responsible for EGFR transactivation (Keates *et al*., 2001; Wallasch *et al*., 2002). Involvement of ADAM17 has been documented in the release of TNF-α and three EGFR ligands, including HB-EGF (sunnarborg *et al*., 2002). Pretreatment with anti-HB-EGF antibody showed no inhibition for the phosphorylation of serine residue of EGFR at 60 min of *H. pylori*-infection (Fig. 5.4A). We next performed flow cytometry in which the cell surface expression of endogenous EGFR could be examined quantitatively. *H. pylori*-induced endocytosis of EGFR was not inhibited by anti-HB-EGF antibody (Fig. 5.4B). However, anti-HB-EGF antibody demonstrated complete inhibition of HB-EGF-induced endocytosis of EGFR (Fig. 5.4C). Collectively, these results vividly pointed out the occurrence of serine phosphorylation of EGFR which is independent of HB-EGF-related transactivation of the receptor.

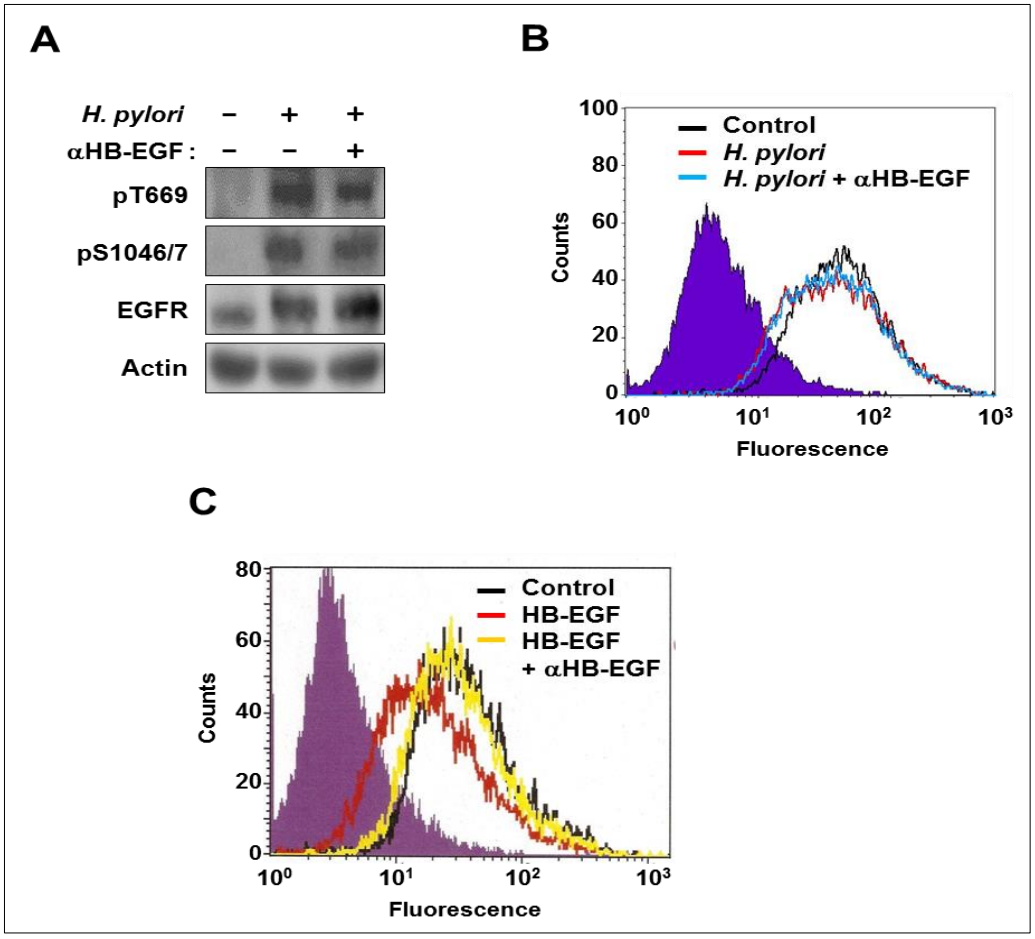

Figure 5.4: Role of HB-EGF in phosphorylation of serine residue and EGFR endocytosis. **A**, AGS cells were cultured in the presence or absence of anti-HB-EGF antibody (3μg/ml) for 30 min followed by addition of *H. pylori* for another 60 min; **B,** Effect of anti-HB-EGF antibody on *H. pylori*-induced endocytosis of EGFR. Gastric cells were pre-incubated with anti-HB-EGF antibody (3μg/ml) for 30 min followed by *H. pylori* infection for 60 min. Blue area shows negative control in the data of flow cytometry. Black, red and blue lines exhibit control, *H. pylori*-infected cells, and *H. pylori*-infected cells pretreated with anti-HB-EGF antibody respectively; **C**, Effect of anti-HB-EGF antibody on HB-EGF-induced endocytosis of EGFR. MKN45 cells were pre-incubated with anti-HB-EGF antibody (3μg/ml) for 30 min followed by HB-EGF for 10 min and subsequent flow cytometric analysis. Purple area shows negative control in the data of flow cytometry. Black, red and yellow lines exhibit control, HB-EGF-treated cells, and HB-EGF-treated cells pre-incubated with anti-HB-EGF antibody respectively. Each figure shown is representative of three independent experiments. Multiplicity of *H. pylori* infection was 50:1.

5.5 Role of TAK1 and p38α in phosphorylation of EGFR serine residue

To further characterize the signaling pathways leading to serine phosphorylation, we next confirmed the role of TAK1 and p38α in phosphorylation of serine EGFR residues and its ultimate internalization. siRNA experiment was performed with siTAK1 and sip38α. As shown in figure 5.5A, siRNA against TAK1 not only inhibited the phosphorylation of EGFR serine but also p38α which reflects the involvement of TAK1 in p38α activation and subsequent inhibition of serine phosphorylation. Similarly, siRNA against p38α markedly decreased the phosphorylation of serine residue (Fig 5.5B). Immunofluorescence staining data showed that EGFR internalization in *H. pylori*-infected TAK1- or p38α-knockdown AGS cells was inhibited, and EGFR protein was localized on the membrane without internalization of EGFR (Fig. 5.5C). These results clearly demonstrated the crucial involvement of TAK1 and p38α in phosphorylation of serine residue of EGFR.

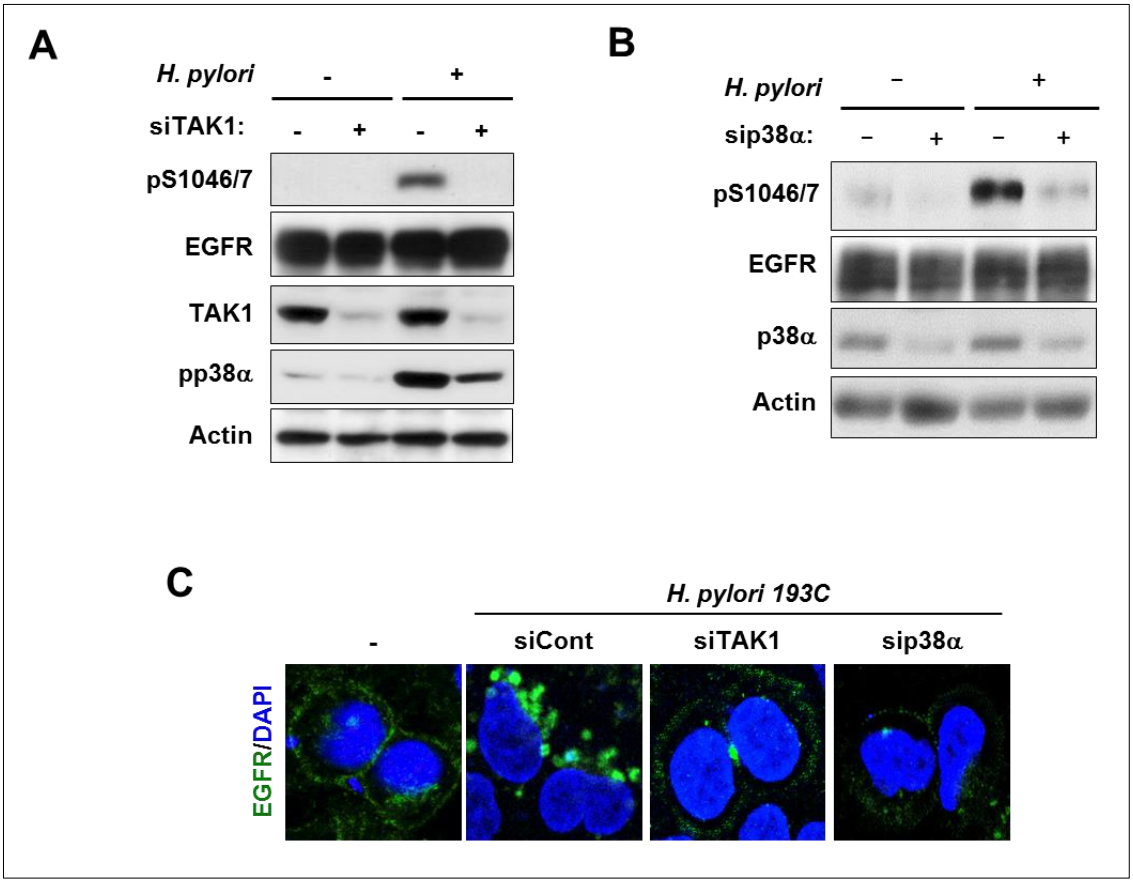

Figure 5.5: Role of TAK1 and p38α in phosphorylation of EGFR serine residue.

A and **B**, AGS cells were transfected with siRNAs against TAK1 (A) and p38α (B) and luciferase as control. At 72 h post-transfection, cells were stimulated with *H. pylori* for 60 min. Whole cell lysates were prepared and immunoblot analysis was performed with indicated antibodies as mentioned before; **C**, Internalization of EGFR was analyzed by immunofluorescence in siTAK1 or sip38α treated gastric epithelial cells at 60min post- *H. pylori* infection.

Multiplicity of *H. pylori* infection was 50:1. Each figure shown is representative of three independent experiments.

5.6 Novel EGFR activation pathway in H. pylori*-infected gastric epithelial cells*

These observations clearly signify the existence of an alternate pathway of EGFR activation via TAK1-p38α phosphorylation of serine residue of EGFR, independent of classical HB-EGF-related transactivation of the receptor (Fig. 5.6).

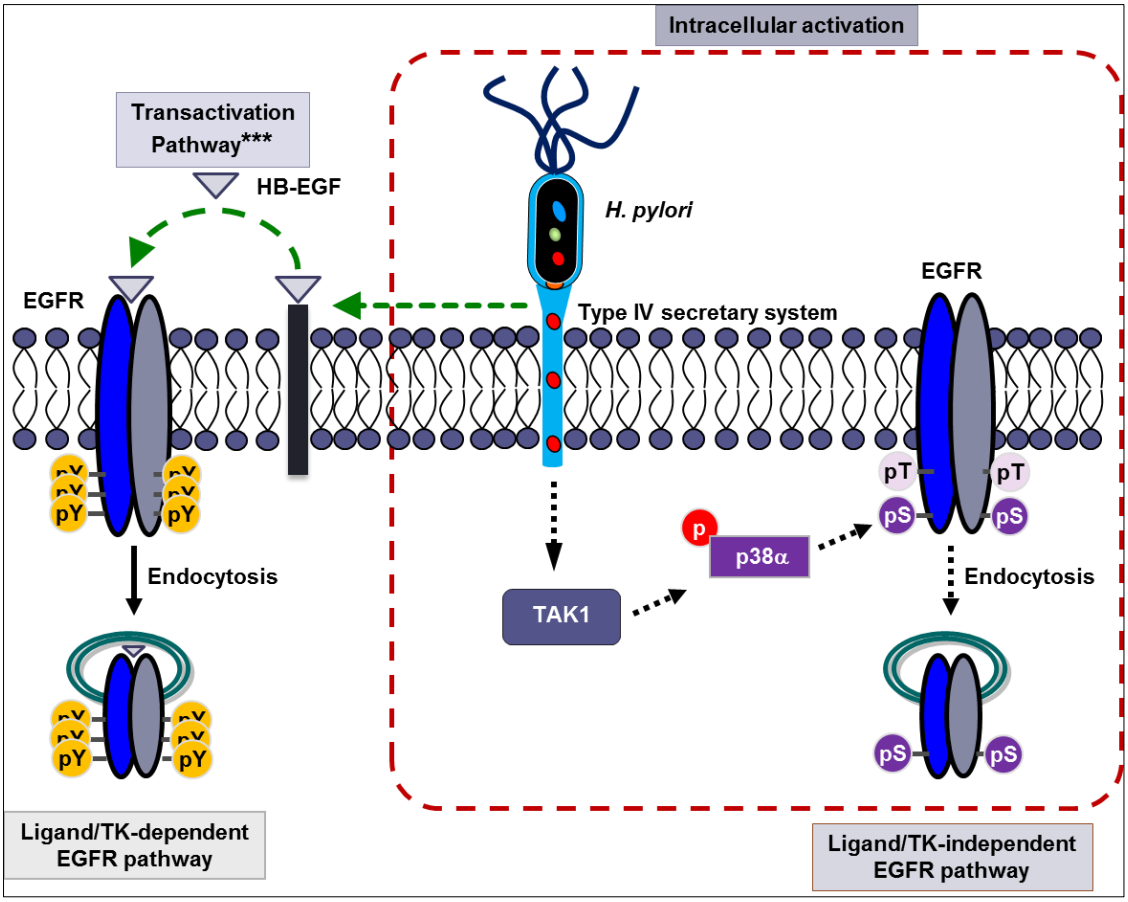

Figure 5.6: Schematic representation of an alternate pathway in *H. pylori*-infected gastric epithelial cells. *H. pylori* activate EGFR via TAK1-p38α phosphorylation of serine residue of EGFR, independent of classical HB-EGF-related transactivation of the receptor. Intracellular pathway shown within red dotted box is reported for the first time in this study.

**** (Transactivation pathway: Keates et. al., J Biol Chem (2001) 276: 48127-34)*

5.7 Time-dependent effect of H. pylori *infection on expression of hBDs*

Next, we assessed the expression of each human β-defensin (hBD1, 2, and 3) in the AGS cell line. RT-PCR data showed that hBD1 was constitutively expressed in uninfected AGS cells and this expression decreased after *H. pylori* infection (Fig. 5.7). Neither hBD2 nor hBD3 was expressed in uninfected AGS cells. However, during the early phase of *H. pylori* infection (up to 2-8 h after co-culture) in AGS cells, expression of hBD2 and of hBD3 was upregulated; interestingly, hBD3 expression was higher than hBD2 expression and time-dependent (Fig. 5.7). In the late phase of infection (48 h), hBD3 release was decreased, thus possibly allowing the *H. pylori* to survive long term in hostile gastric environment. Previous studies show that the anti-*H. pylori* activity of hBD3 is 100 times more potent than that of hBD2 (Kawauchi *et al*., 2006; Bauer *et al*., 2012); therefore, the precise cellular mechanism of hBD3 release is very important to understanding how *H. pylori* evade host defense responses and survive in the stomach.

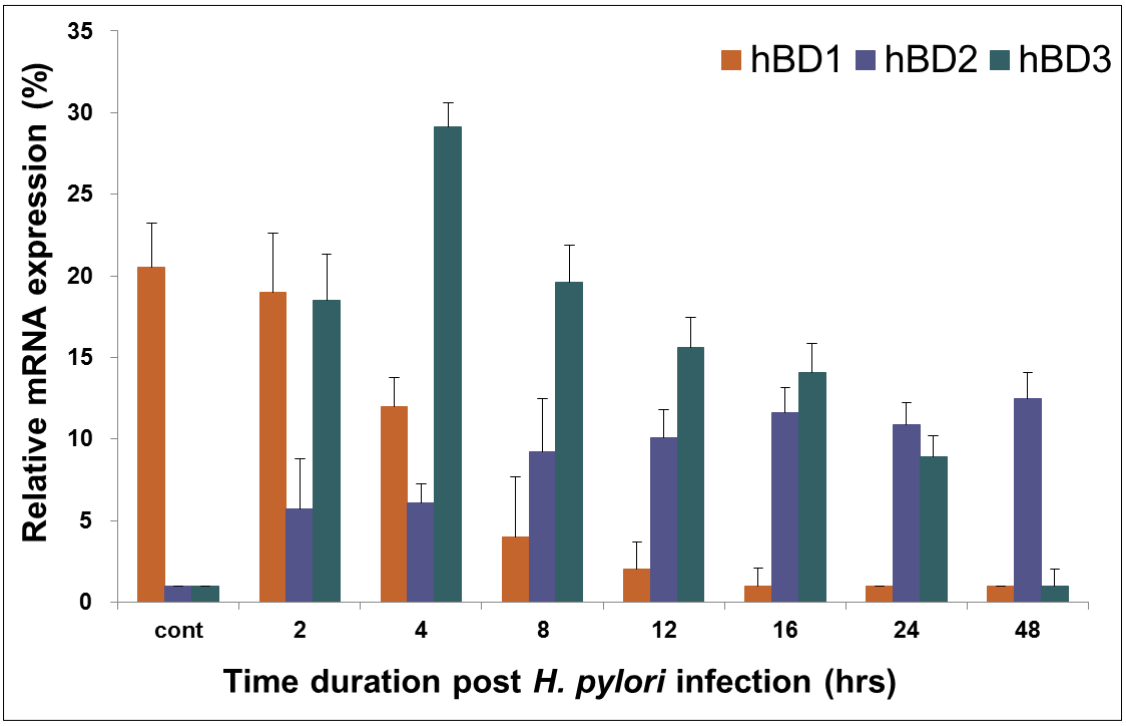

Figure 5.7: Time dependent effect on expression of human β-defensins-1, -2, and -3 in *H. pylori*-infected gastric cells (AGS).

The hBD1 is constitutively expressed in human gastric cells and is suppressed completely after 24 h post-infection. The hBD2 and 3 are released upon *H. pylori* 193C infection as a host immune response. The hBD2 seems to maintain fairly low constant levels for up to 48 h after *H. pylori* infection, but hBD3 expression increased to very high levels in first 4 h then decreases with time and reaches to uninfected levels at 48 h after *H. pylori* infection. Multiplicity of infection was 50 bacteria to each cell (MOI; 50:1). Data is represented as mean ± SEM of three independent experiments (n=3).

5.8 Release of hBD3 from gastric epithelial cells infected by wild-type H. pylori *and CagA knocked-out* H. pylori

In order to evaluate the time-dependent release of hBD3, two wild-type strains of *H. pylori* and a CagA^{-} strain were used to induce hBD3 expression in AGS and MKN45 cell lines. Based on RT-PCR data, each cell type exhibited a time-dependent increase in hBD3 expression after infection with either of two wild-type *H. pylori* clinical strains (193C or CPY2052). With each *H. pylori* strain, hBD3 mRNA expression peaked at 8 h after *H. pylori* infection. Thereafter, hBD3 mRNA levels began to decline, and no hBD3 expression was evident 48 h after *H. pylori* infection. In contrast, infection with *H. pylori* CagA knocked-out strain, hBD3 mRNA expression was only weakly downregulated during the late phase of infection (Fig. 5.8A). For each *H. pylori* strain hBD3 protein expression peaked 8-24 h after *H. pylori* infection. Similar to mRNA expression, hBD3 protein expression was also downregulated during the late phase of *H. pylori* infection. However, hBD3 protein expression, like the mRNA, persisted in both AGS and MKN45 cell lines infected with CagA knockout *H. pylori* and was only slightly down regulated during the late-phase infection (Fig. 5.8B and 5.8C). These results indicated that release of hBD3 during early-phase infection was CagA-independent. We also analyzed *H. pylori* lysates for cross-reactivity between *H. pylori* antigens and hBD3 antibody. The hBD3 antibody used in this study did not cross-react with any *H. pylori* proteins in any of the four strains (see *supplementary figure 2, page 69*).

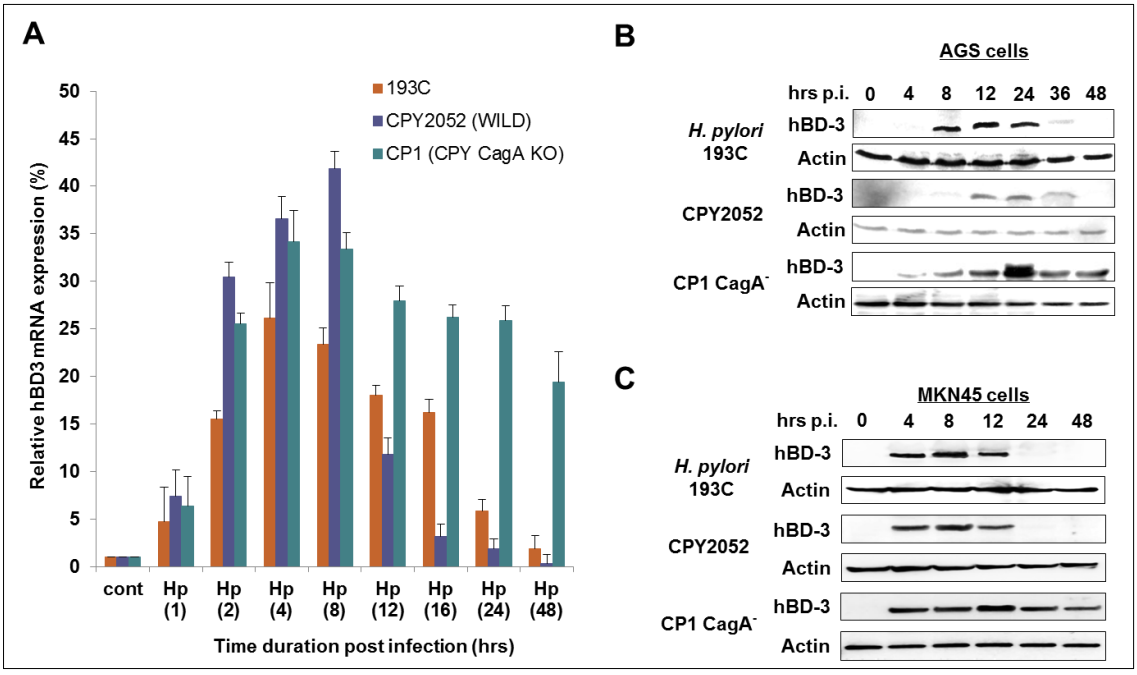

Figure 5.8: Time dependent effect of *H. pylori* infection on human beta defensin-3 (hBD-3) release from human gastric epithelial cells.

A, The ability of *H. pylori* strain 193C, CPY2052 and CP1 (CagA^{-} for CPY2052) to induce hBD3 mRNA expression in AGS cells was examined. *H. pylori* 193C and CPY2052 induced time-dependent increases in hBD3 expression for 8 h after co-culture; expression then decreased and became negligible at 48 h after co-culture. However, hBD3 expression was not down-regulated 8 h or later after co-culture with *H. pylori* CP1 (CagA^{-}). RT-PCR data is represented as mean ± SEM of three independent experiments (n=3); **B**, In AGS cells the hBD3 protein was detectable 4 h after co-culture with each individual strain and showed a pattern similar to that of mRNA; **C**, Similar pattern of hBD3 protein expression was confirmed in MKN-45 cell line infected with three strains of *H. pylori*.

Each western blot shown is representative of three independent experiments. Multiplicity of infection was 50 bacteria per cell for all experiments (MOI; 50:1).

5.9 The effect of exogenous EGF on hBD3 release

EGF, a ligand of EGFR, is known to cause auto-phosphorylation and internalization of EGFR via classical transactivation pathway leading to activation of MAPK pathways (Morandell *et al*., 2008). To evaluate the effect of exogenous EGF on the release of hBD3 in gastric epithelial cells, AGS cells were pre-incubated with EGF (10 ng/ml) for 5 min and then incubated with or without *H. pylori* (193C) infection for 8 h; hBD3 release was analyzed. EGF alone activated EGFR, but failed to induce hBD3 release from AGS cells. In contrast, *H. pylori* (193C) infection even without the EGF stimulation was able to induce high levels of hBD3; moreover, there was no significant additive effect of EGF and *H. pylori* infection together (Fig. 5.9A). Also when the AGS cells were treated with high doses of exogenous EGF, the hBD3 was not induced from AGS cells (Fig. 5.9B). Therefore, this data excludes the role of classical transactivation pathway in the release of hBD3 from *H. pylori*-infected gastric epithelial cells.

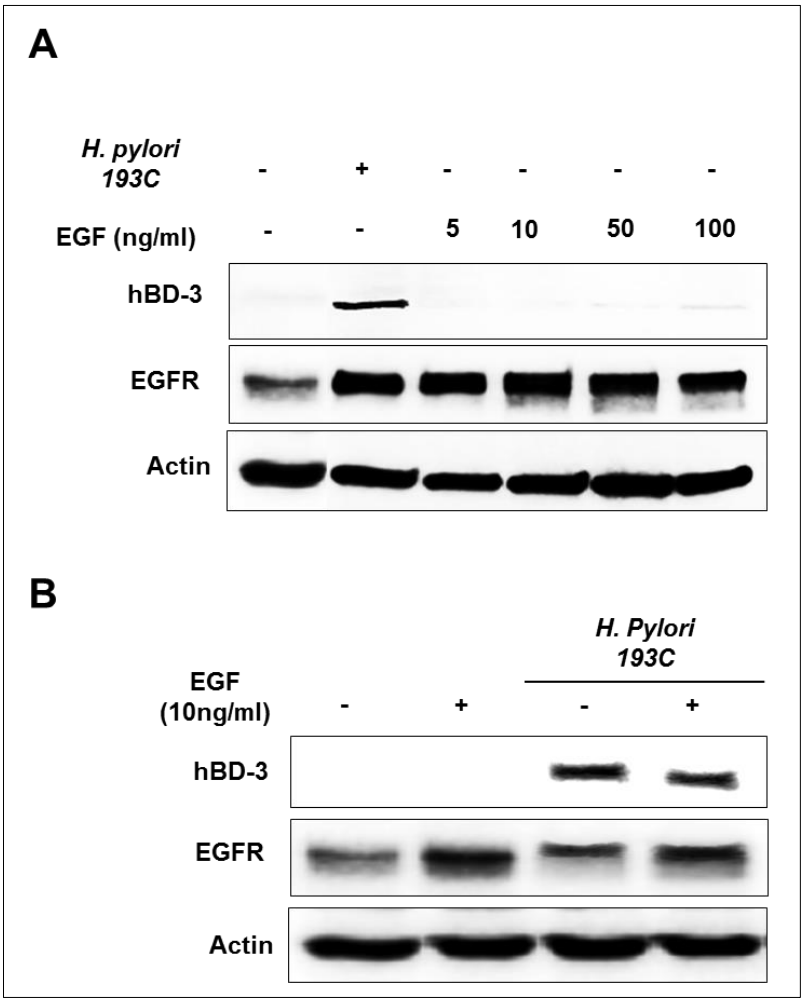

Figure 5.9: Effect of EGF on hBD3 release from *H. pylori*-infected gastric epithelial cells.

A, AGS cells were co-cultured with *H. pylori* for 8 h with or without a 5 min EGF (10 ng/ml) pretreatment. Pretreatment with EGF alone activated EGFR, but did not induce hBD3 release. This finding showed that activation of EGFR by *H. pylori* was essential for hBD3 release from gastric epithelial cells; **B**, Dose-response effect of EGF treatment on release hBD3 release from *H. pylori*-infected gastric epithelial cells was evaluated. For positive control, AGS cells were co-cultured with *H. pylori* 193C. Samples were collected 8 h after experiment.

Each figure shown is representative of three independent experiments. Multiplicity of *H. pylori* infection was 50:1.

5.10 Effect on the hBD3 release by inhibiting TAK1 or p38α

TAK1 is a key regulator in the phosphorylation and activation of p38α, which leads to EGFR activation via phosphorylation of EGFR serine residues in HeLa cells (Nishimura *et al*., 2009) and we showed that similar EGFR activation pathway exists in *H. pylori*-infected gastric epithelial cells; however, this pathway is not proven on the functional linkage with the release of hBD3 in *H. pylori*-infected gastric epithelial cells. Therefore, next we evaluated the direct role of the TAK1-p38α pathway in the induction of hBD3 from *H. pylori*-infected gastric epithelial cells. To investigate the roles of TAK1 and p38α in hBD3 release, gastric epithelial cells were pre-incubated with SB203580 (SB), a p38α inhibitor, or 5Z-7-oxozeaenol (5OZ), a TAK1 inhibitor, for 30 min before co-culture with *H. pylori* 193C. In the presence of either inhibitor (SB or 5OZ), hBD3 mRNA expression was significantly downregulated ($p < 0.01$) (Fig. 5.10A). Next, we demonstrated that hBD3 protein expression was also downregulated in the presence of either TAK1 or p38α inhibitor in gastric epithelial cells 8 h after infection with either of two *H. pylori* strains, 193C or CPY2052 (Fig. 5.10B).

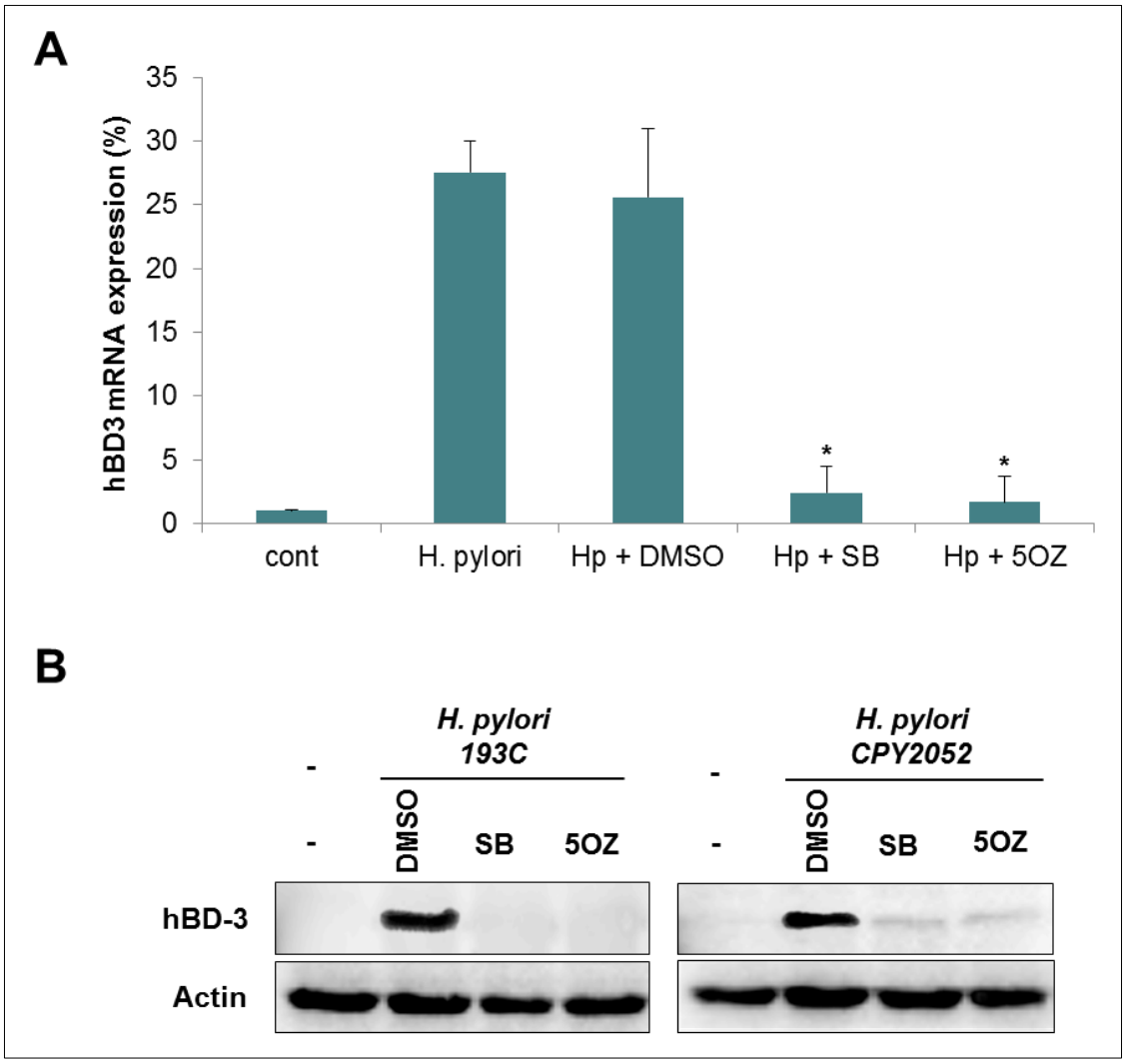

Figure 5.10: Effect on the hBD3 release by inhibiting TAK1 or p38α *H. pylori*-infected gastric epithelial cells.

A, to evaluate the role of TAK1 and p38α we performed experiments with a p38α inhibitor (SB203580; SB) or a TAK1 inhibitor (5Z-7-oxozeaenol; 5OZ). RT-PCR analysis of hBD-3 release 2 h after co-culture with *H. pylori* 193C in the presence of SB or 5OZ confirms roles for TAK1 and p38α in hBD3 release. (n=3); **B**, Expression of hBD3 8 h after co-culture with *H. pylori* 193C and CPY2052 analyzed by western blot in the presence of all inhibitors. Figure is representative of three independent experiments.

Multiplicity of *H. pylori* infection was 50:1. RT-PCR data is represented as mean ± SEM of three independent experiments. (*, $p < 0.01$)

5.11 Role of T4SS machinery in the release of hBD3 from H. pylori-*infected gastric cells*

In order to evaluate the role of T4SS machinery of *H. pylori* in the release of hBD3, AGS cells were infected for 45 min or for 8 h with wild-type *H. pylori* (193C) or T4SS mutant (△*virB4*) *H. pylori* strain. Infection with a *H. pylori* △virB4 (impaired T4SS) strain did not induce hBD3 protein expression in AGS cells at any time point. Also, *H. pylori* △virB4 was unable to induce the phosphorylation of TAK1 or p38α (Fig. 5.11A). This data along with the results shown in figure 8 indicated that release of hBD3 during early-phase infection was T4SS-dependent, but was CagA-independent.

Along with CagA, *H. pylori* is also capable of delivering peptidoglycan via T4SS into the host cells. Delivery of *H. pylori* peptidoglycan by T4SS activates NF-κB and MAPKs via NOD1 and it induces IL-8 production from gastric epithelial cells (Allison *et al*., 2009; Viala *et al*., 2004). Therefore, to exclude the role of NOD1 activation in the release of hBD3 by *H. pylori* peptidoglycan, NOD1-knockdown gastric epithelial cells were infected with *H. pylori* 193C. After 8 h of *H. pylori* co-culture, hBD3 was induced in both types of gastric epithelial cells, wild-type AGS cell and NOD1-knockdown AGS cells (Fig. 5.11B). This data suggested that the production of hBD3 was dependent on EGFR activation, but independent of NOD1 activation.

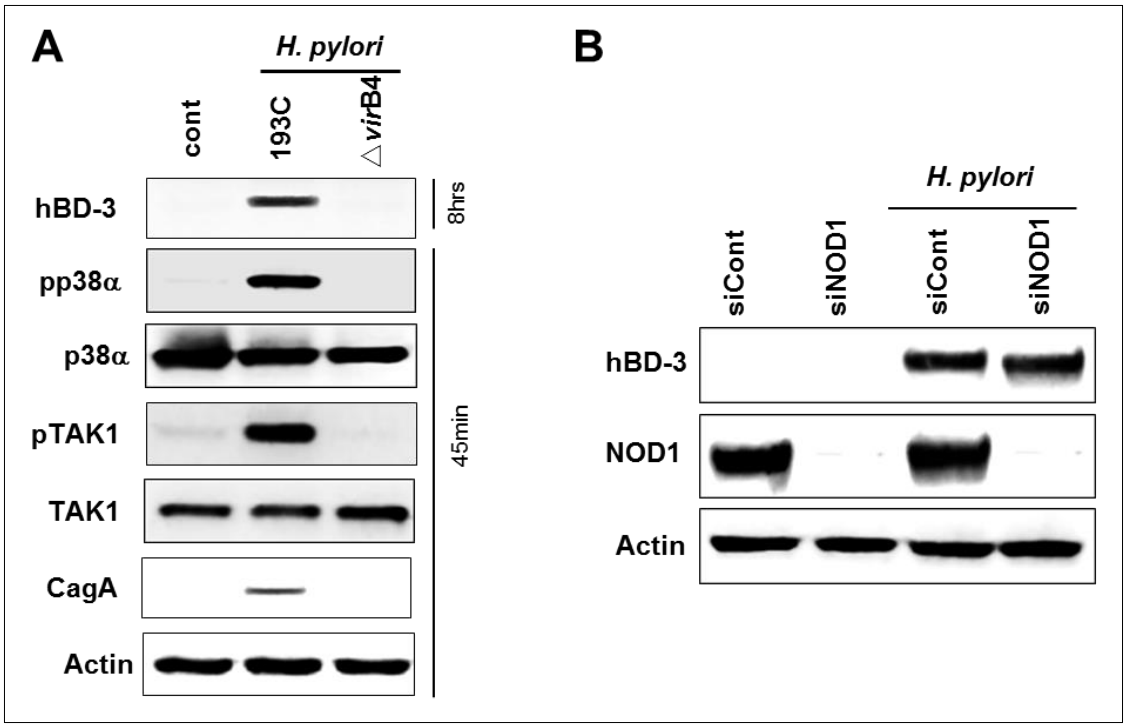

Figure 5.11: Role of type IV secretary system machinery in the release of hBD3 from *H. pylori*-infected gastric epithelial cells.

A, AGS cells were infected *H. pylori* △*vir*B4 for 45min and for 8 h, separately. *H. pylori* △*vir*B4, which harbor impaired T4SS, did not activate TAK1 and p38α, also failed to induce hBD3 release from the host cells. Co-culture with *H. pylori* △ *vir*B4 does not express intracellular CagA. To ensure expression of intracellular CagA, co-cultures cells were rigorously washed for 6 times using ice-cold PBS before obtaining whole cell lysates; **B**, AGS cells were transfected with siRNAs for NOD1. Approximately 72 h after transfection, the cells were co-cultured with *H. pylori* for 8 h. knockdown of NOD1 from gastric epithelial cells had no effect on the release of hBD3.

Multiplicity of *H. pylori* infection was 50:1. Each figure shown is representative of three independent experiments.

5.12 Effects of siEGFR, siTAK1, or sip38α on hBD3 release from H. pylori*-infected gastric cells*

To evaluate the role of EGFR, TAK1 and p38α activation in *H. pylori*-induced hBD3 release, we analyzed hBD3 release in EGFR-, TAK1- or p38α-knockdown AGS cells. Release of hBD3 was inhibited in EGFR knockdown gastric cells infected with *H. pylori* 193C (Fig. 5.12A). Furthermore, knockdown of TAK1 or p38α in AGS cells completely inhibited the phosphorylation of the Ser-1046/7 residue of EGFR. Also, none of the EGFR tyrosine residues were phosphorylated in either *H. pylori*-infected control AGS cells or siRNA knockdown AGS cells (Fig. 5.12B). EGFR tyrosine phosphorylation was detected using exogenous EGF (10 ng/ml) treated AGS cells as a positive control for this experiment. Next, we showed that hBD3 release of was completely inhibited in siTAK1 or sip38α *H. pylori*-infected AGS cells (Fig. 5.12C).

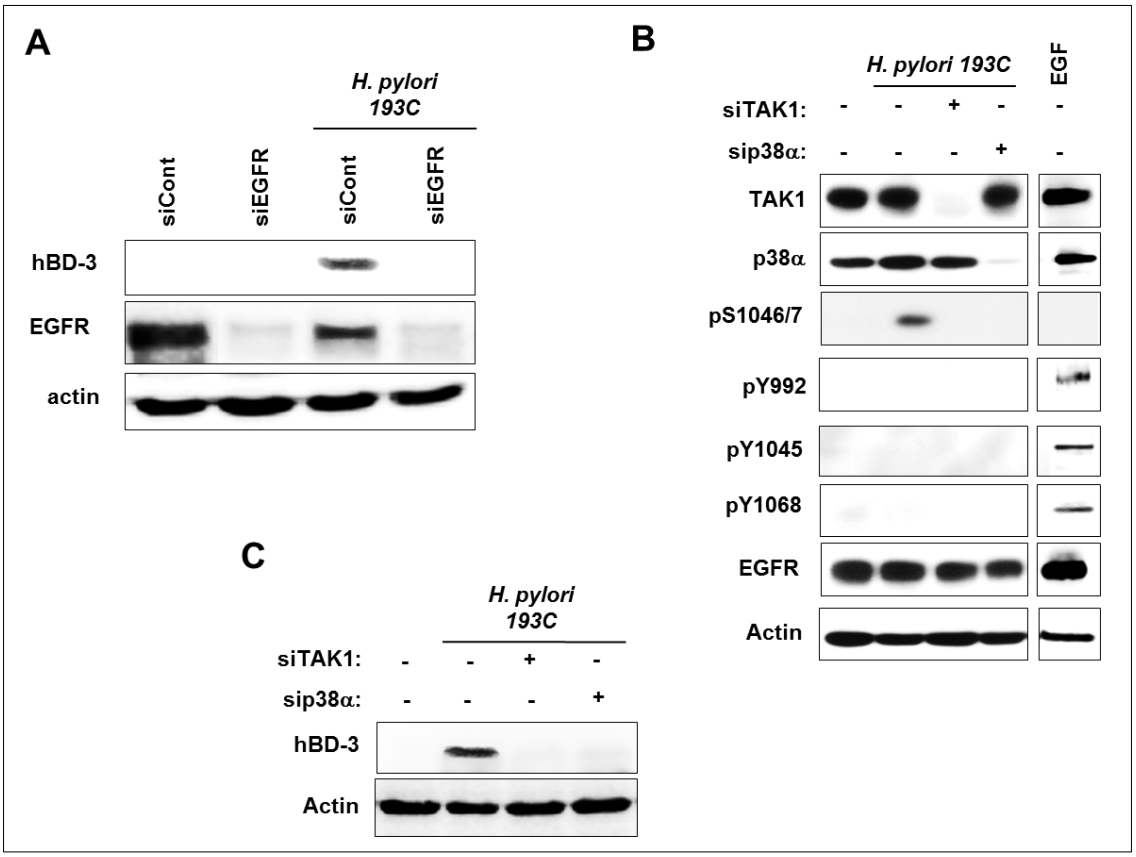

Figure 5.12: Effect of siEGFR, siTAK1, and sip38α on release of hBD3 from *H. pylori*-infected gastric epithelial cells.

A, AGS cells were transfected with siRNAs for EGFR. Approximately 72 h after transfection, the cells were co-cultured with *H. pylori* for 8 h. Knockdown of EGFR in gastric epithelial cells inhibited release of hBD3; **B**, AGS cells were transfected with siRNAs for TAK1, or p38α. Approximately 72 h after transfection, the cells were infected with *H. pylori* for 60 min. Phosphorylation of serine residue of EGFR was not seen in siTAK1 and sip38α gastric epithelial cells. Also, these results show that during early *H. pylori* infection phosphorylation EGFR tyrosine resides was not involved in activation of TAK1-p38α pathway. AGS cells treated with EGF (10 ng/ml) for 60 min was used as positive control; **C**, Induction of hBD3 was completely inhibited in siTAK1 or sip38α cells.

Multiplicity of *H. pylori* infection was 50:1. Each figure shown is representative of three independent experiments.

5.13 Induction of hBD3 via novel EGFR activation pathway in H. pylori*-infected gastric epithelial cells*

Hence, these results suggested that *H. pylori* infection in gastric epithelial cells activated a TAK1-p38α pathway and caused phosphorylation of a specific EGFR serine residue to induce hBD3 production (Fig. 5.13).

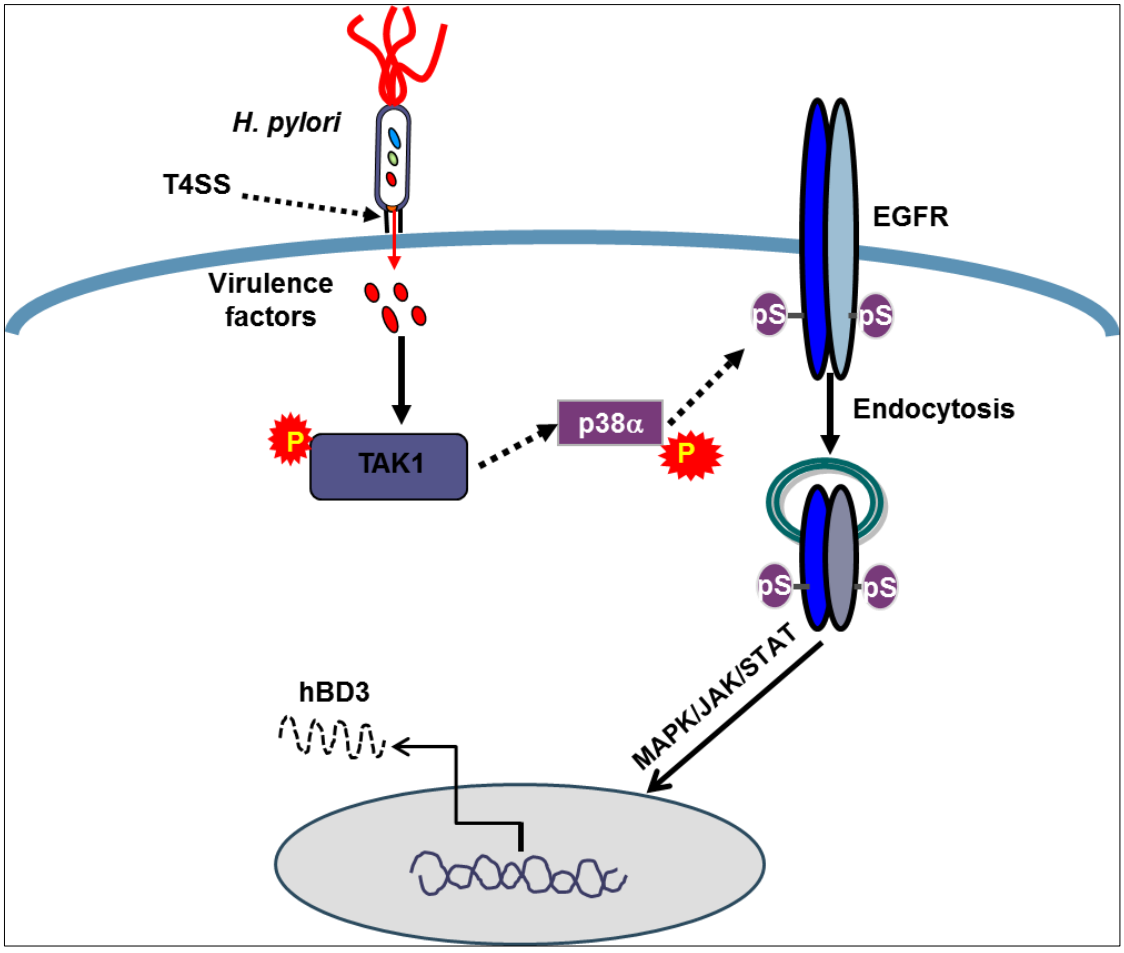

Figure 5.13: Schematic representation of release of hBD3 from gastric epithelial cells by novel EGFR activation pathway in early-phase of *H. pylori* infection.

Helicobacter pylori: *H. pylori*, T4SS: type IV secretion system machinery, EGFR: endothelial growth factor receptor, pS: phosphorylated serine residue of EGFR, hBD3: human β-defensin 3.

CHAPTER 6

6.1 DISCUSSION

H. pylori activate several intracellular signaling pathways in the host cell, and this signaling activates host innate immune responses (D'Elios and Czinn, 2014). AMPs released in response to *H. pylori* infection play an important role in cellular defense mechanisms. However, *H. pylori* is able to escape or overcome these host defense mechanisms (Aebischer, Meyer and Anderson, 2010; George *et al*., 2003). Therefore, understanding cellular pathways involved in the release of AMP is vital to developing treatment strategies for *H. pylori*-related diseases. In the present study, we have demonstrated for the first time that in *H. pylori*-infected cells, EGFR can be regulated via intracellular pathways which are independent of the ligand- based (through HB-EGF) transactivation of the receptor. Also, we demonstrated that the release of hBD3 from *H. pylori*-infected gastric epithelial cells occurs via a novel EGFR-activating pathway during an early phase of *H. pylori* infection.

Role of *H. pylori* has been well recognized as class I carcinogen, by World Health Organization, in the development of gastric cancer (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (Ed.), 1994). However, the pathogenesis behind *H. pylori*-induced carcinogenesis is intricate and poorly understood. Simultaneous involvement of several factors and signaling pathways, which are sometimes contradictory or conflicting to each other, makes the process complicated (Schweitzer *et al*., 2010; Tegtmeyer *et al*., 2009). A recent such report has shown that simultaneous activation of EGFR protects gastric epithelial cells from *H. pylori*-induced apoptosis which may heighten the risk for the development of gastric cancer (Yan *et al*., 2009). EGFR

overexpression is common in human gastric cancer and high EGFR levels have been correlated with poor prognosis (Normanno *et al*., 2006). *H. pylori* not only up-regulates EGFR expression but also cause transactivation of EGFR via HB-EGF (Keates *et al*., 2001). Several reports have also documented the activation of EGFR via tyrosine kinase dependent auto-phosphorylation of different tyrosine residues upon dimerization with ligands such as EGF which triggers mitogen activated protein (MAP)-kinase signaling pathways (Keates *et al*., 2001; Morandell *et al*., 2008; Nakakuki *et al*., 2008). We have also demonstrated here that both EGF and HB-EGF induced tyrosine phosphorylation in AGS cells. Interestingly, EGF, HB-EGF, TNF-α, and *H. pylori* all exhibited phosphorylation of threonine residue of EGFR which signifies that several pathways can modulate threonine phosphorylation. However, there was no effect of either HB-EGF or EGF on phosphorylation of serine residue of EGFR which appeared quite specific to *H. pylori* infection. This encouraged us to undermine the cause and role of serine phosphorylation in EGFR activation and internalization. We showed that *H. pylori* infection clearly induced phosphorylation of TAK1 which in turn causes activation of p38α. Activation of p38α phosphorylates serine residue of EGFR and subsequently induces internalization of the receptor in p38α dependent manner. We did not observe phosphorylation of tyrosine residues in response to *H. pylori* at 60 min of co-culture. We could not rule out the possibility that this difference may be caused by difference in duration of *H. pylori* infection or multiplicity of infection. We further demonstrated that pretreatment with anti-HB-EGF neutralizing antibody did not affect the p38α-induced phosphorylation and internalization of

EGFR (Fig. 4). These observations clearly signify the existence of alternate pathway of EGFR activation via TAK1-p38α phosphorylation of serine residue of EGFR, independent of classical HB-EGF-related transactivation of the receptor.

Binding of the ligand to extracellular domain of EGFR or *H. pylori* infection triggers EGFR activation (Keates *et al*., 2001; Keates *et al*., 2007; Zhang *et al*., 2006). In case of *H. pylori* infection, several virulence factors like CagA or VacA has been linked with severe outcomes of infection. CagA has long been under debate for its controversial role in *H. pylori*-associated pathology. For instance, contrary reports are documented on the role of CagA in the activation of NFκB (Lamb *et al*., 2009; Sokolova *et al*., 2014; Schweitzer *et al*., 2010). Similarly, previous reports also demonstrated that CagA protein itself does not induce inflammatory responses, and play no role in MAP kinase activation (sokolova *et al*., 2014; Keates *et al*., 1999). EGFR overexpression and activation has been well documented by *H. pylori*. As far as our new pathway of EGFR internalization is concerned, CagA does not appear to play role in inducing serine phosphorylation of EGFR and eventually no effect on EGFR internalization (Fig. 4D). On the other hand, CagA has been shown to selectively inhibit EGFR endocytosis on prolong incubation i.e. after 20 h of co-culture (Bauer, Bartfeld and Meyer, 2009).

As reported earlier, *H. pylori* infection can lead to the release of membrane bound HB-EGF, hence two EGFR activation pathways can occur in the infected gastric mucosa; one is classical transactivation via HB-EGF and other by novel intracellular phosphorylation of EGFR via TAK1-p38α activation. The dilemma of simultaneous modulation of several pathophysiological

pathways by *H. pylori* renders outcome complicated and unpredictable. It's too early to conclude which pathway of EGFR activation comes first or which pathway play the major role in pathogenicity of *H. pylori* infection but from our preliminary data we might postulate that intracellular TAK1-p38α pathway may have impact on EGFR activation in early phase of infection while the classical pathway of HB-EGF play role in chronic phase. However, this novel EGFR activation pathway and the interaction between TAK1 and EGFR by *H. pylori* might help in understanding the complex pathogenic process by this class I carcinogenic bacterium. Therefore, next we aimed to determine the functional role of this novel pathway in relation to the release of hBDs (hBD1, 2, and 3) from gastric epithelial cells as a host-immune response against *H. pylori* infection.

HBD1 is constitutively expressed in epithelial cells, and it has been reported that genetically modified hBD1-knockedout mice have an impaired ability to combat bacterial infections (Morrison *et al*., 2002). In fact, we also found that hBD1 was constitutively expressed in AGS cells, and this expression was completely suppressed after *H. pylori* infection. This suppression was due to CagPAI-dependent activation of NF-κB pathway in *H. pylori* infected gastric cells (Patel *et al*., 2013). Further, our results and some previous *in vitro* findings showed that hBD2 and hBD3 expression was increased upon *H. pylori*-infection of gastric epithelial cells (Boughan *et al*., 2006; Bauer *et al*., 2013; Patel *et al*., 2013). Some studies have shown that NOD1 activation following cagPAI^{+} *H. pylori* infection is a key regulator of hBD2 release (Boughan *et al*., 2006; Patel *et al*., 2013) and that EGFR-dependent activation of MAP kinase and JAK/STAT

signaling induces hBD3 release by *H. pylori* infection (Bauer *et al*., 2012). However, the precise EGFR activation mechanism was not investigated. Our study is the first report that documents the exact molecular mechanism that is responsible for hBD3 release from *H. pylori*-infected gastric epithelial cells; this mechanism involved a TAK1-p38α activation pathway that led to phosphorylation of specific EGFR serine residue and subsequent EGFR internalization.

During the late-phase of *H. pylori* infection, CagA binds to SHP-2 and dephosphorylates EGFR to suppress hBD3 expression; this suppression allows *H. pylori* to evade host immune responses (Bauer *et al*., 2012). Also in order to evade immune response, cagPAI^{+} *H. pylori* infection can induce the overexpression of programmed death-1 ligand 1 (PD-L1) in host gastric epithelial cells to suppress T-cell activation. This *H. pylori*-mediated PD-L1 expression was regulated by bacterial peptidoglycan and only partially dependent on CagA injection into the host cell (Das *et al*., 2006; Lina *et al*., 2015). In this study, we showed a time-dependent increase in hBD3 expression in the early phase of *H. pylori* infection with a CagA-knockout strain; therefore our data suggested that hBD3 release in early phase infection was independent of the presence of CagA in *H. pylori*.

Delivery of *H. pylori* peptidoglycan by T4SS activates NF-κB and MAPKs via NOD1 and it induces IL-8 production from gastric epithelial cells (Allison *et al*., 2009; Viala *et al*., 2004). We showed that hBD3 release was induced in NOD1-knockdown gastric epithelial cells infected with *H. pylori*; therefore, the peptidoglycan-NOD1 pathway did not have any role in the

activation of TAK1-p38α pathway in *H. pylori*-induced hBD3 release from gastric epithelial cells. Based on these results, other unknown virulence factors besides CagA or peptidoglycan might be involved in the induction of hBD3, although the presence of functional T4SS in *H. pylori* was essential to induce hBD3 production.

Multiple EGFR tyrosine residues are phosphorylated when gastric cells are co-stimulated with EGF in the presence of *H. pylori* (Bauer *et al*., 2013; Bauer *et al*., 2012) or co-cultured with *H. pylori* for very long time period (e.g. 24 h) (Bauer *et al*., 2013; Hubbard *et al*., 2007). However, *H. pylori* or TNFα alone fails to induce EGFR tyrosine residue phosphorylation within 60 min after co-culture (Bauer *et al*., 2012; Nishimura *et al*., 2009). Here we showed that only serine (pS1046/7) residue of EGFR was phosphorylated at 60 and 120 min after co-culture with either wild-type or a CagA knockout *H. pylori* strain.

H. pylori infection transactivates EGFR by a disintegrin- and metalloprotease (ADAM17)-dependent, NF–κB-mediated shedding of membrane-bound HB-EGF (Saha *et al*., 2010). Reportedly, EGFR transactivation via membrane-bound HB-EGF induces hBD3 gene expression in a model of skin inflammation (Sørensen *et al*., 2005). However, Boughan *et al*. reported that the expression of hBD3 was only partially decreased (< 50%) even if HB-EGF transactivation pathway was inhibited (Boughan *et al*., 2002), suggesting that some other pathway might be responsible for hBD3 release.

In the present study, we used protein-specific inhibitors and TAK1 or p38α knockdown AGS cells to show that a TAK1-p38α pathway driven EGFR internalization was necessary in the release of hBD3 from *H. pylori*-infected

cells. Notably, our results showed almost complete suppression of hBD3 production when the TAK1-p38α pathway was inhibited by specific chemical inhibitors or by siRNA-medicated knockdown of TAK1 or p38α. We also showed that this TAK1-p38α pathway was dependent on functional *H. pylori* T4SS.

H. pylori obtained from the Japanese population, irrespective of associated disease condition, have similar CagA proteins and EPIYA motif (Yamada *et al*., 2012). Although the two strains used in this study were derived from patients with different diseases, both strains possess a similar EPIYA motif (ABD type). Also we showed that the production of hBD3 in *H. pylori*-infected gastric epithelial cells was independent of CagA in this study. However, the lack of availability of a single, isogenic strain for both CagA and T4SS mutants is a limitation of our study.

We showed that in *H. pylori*-infected gastric cells, the TAK1-mediated phosphorylation of an EGFR serine residue was found to be independent of HB-EGF related transactivation of EGFR. EGFR activation was specific to phosphorylation of serine residue of EGFR in *H. pylori*-infected gastric epithelial cells. Further, hBD3 production was completely inhibited in *H. pylori*-infected EGFR knockdown cells, suggesting that EGFR itself is essential for the release of hBD3 from gastric epithelial cells.

6.2 CONCLUSION

In conclusion, we demonstrated novel signaling pathways to EGFR via TAK1 and MAPKs in *H. pylori*-infected cells. Activation of two functionally distinct pathways, NF-κB pathway and EGFR pathway, via TAK1 might explain the complicated and simultaneous occurrence of wide variety of signals in *H. pylori*-infected cells leading to the dysregulation of cell survival and death. Also, we have demonstrated that this novel TAK1-p38α pathway induced the phosphorylation of an EGFR serine residue, and this pathway functioned in the release of hBD3 from *H. pylori*-infected gastric epithelial cells. This study showed the mechanism by which *H. pylori* infection stimulated hBD3 expression in gastric epithelial cells during the early phase of infection as a host-cell immune response. Such precise molecular studies examining host-pathogen interactions provides an understanding of how *H. pylori* survives and persists in the human gastric mucosa, and studies like this might help in the identification of target molecules for the development of effective strategies to overcome persistent *H. pylori* infection.

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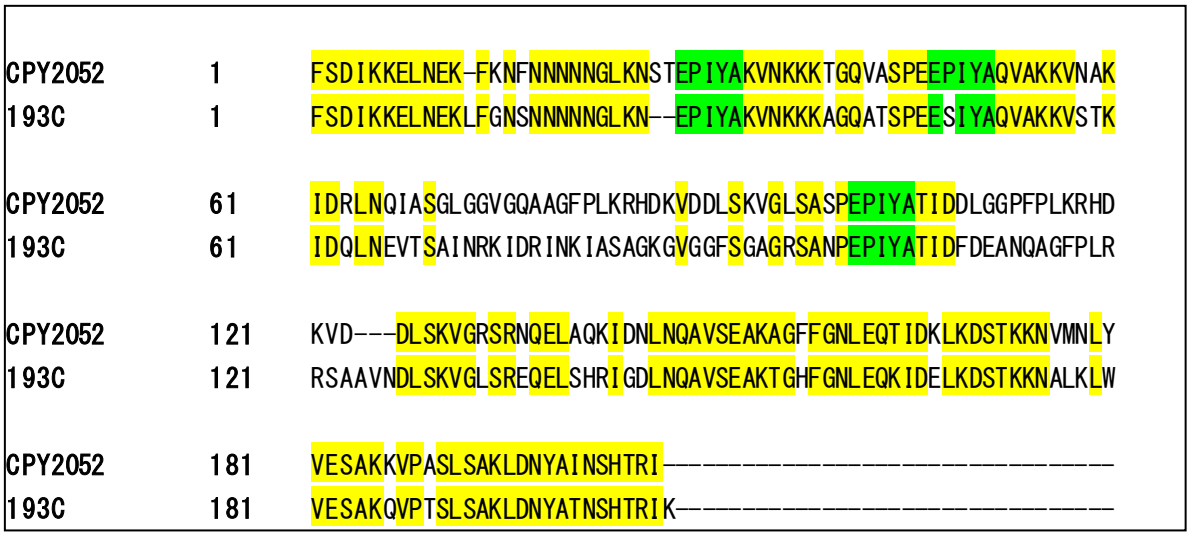
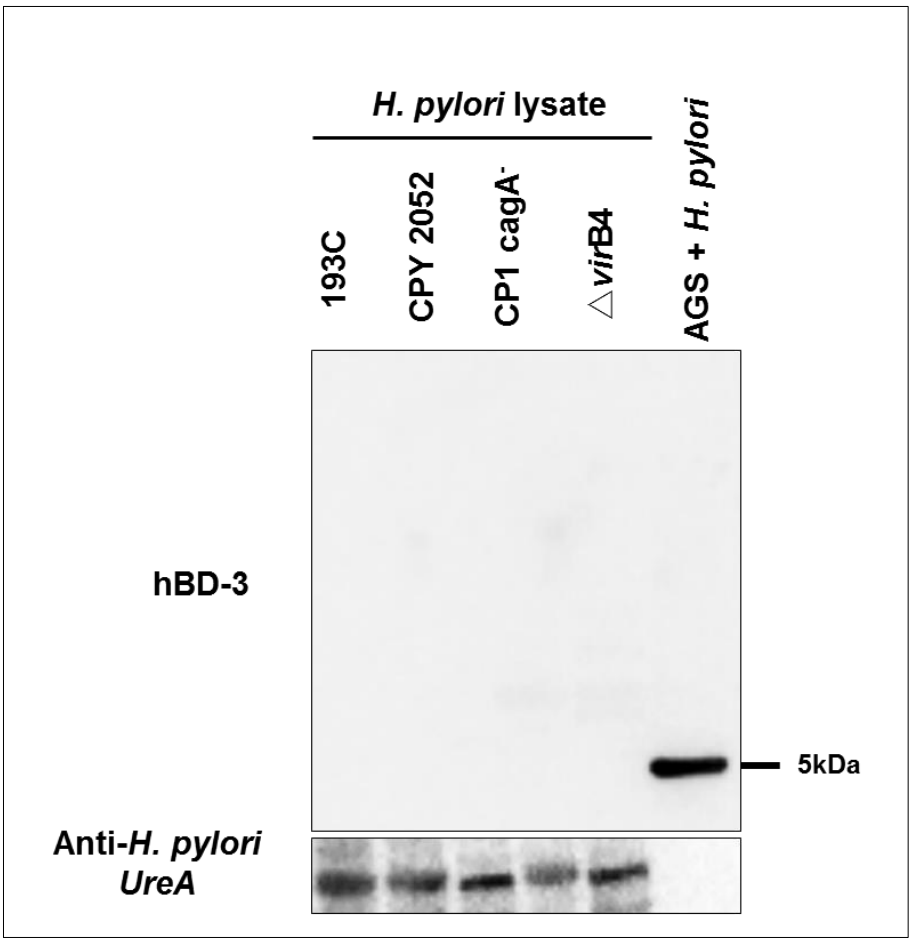
SUPPLEMENTARY FIGURES



Supplementary Figure 1: The amino acid sequence alignment of the two wild-type *H. pylori* strains used in this study. We performed genome sequencing of 3' region of CagA for both the wild-type strains of *H. pylori* (193C and CPY2052) used in this study. Later the genome sequence was translated into amino acid sequence. As both the strains were derived from Japanese patients, the 3' region of CagA of each strain showed similar EPIYA-ABD motif (green highlight).



Supplementary Figure 2: Cross-reactivity between *H. pylori* antigens and hBD3 antibody used in this study. Bacterial protein was extracted using B-PER from each *H. pylori* strain to evaluate presence of CagA protein. Anti-*H. pylori* UreA antibody was used as an internal control. The hBD3 antibody used in this study did not cross-react with any *H. pylori* proteins in all of the four strains used in this study.

THE END