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Helicobacter pylori (H. pylori) Induces Snail Expression via GSK-3β Signaling in Human Gastric Cancer Cells

2013 년 8 월

서울대학교 대학원

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지도교수 서영준

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2013 년 8 월

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Abstract

Helicobacter pylori Induces Snail Expression via GSK-3β Signaling in Human Gastric Cancer Cells

Ngo Hoang Kieu Chi

Under the supervision of professor Young-Joon Surh

Pharmaceutical Bioscience Major, College of Pharmacy

Seoul National University

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related mortality worldwide. Infection with a gastric-specific pathogen *Helicobacter pylori* (*H. pylori*) is the principal factor implicated in the etiology of gastric cancer. Snail has been reported to provoke changes in the cell shape or morphogenetic movement. In the present study, we found that *H. pylori* infection resulted in morphological changes as well as disruption of interaction

between human gastric cancer AGS cells. Moreover, *H. pylori* treatment induced up-regulation of Snail expression through inactivation of glycogen synthase kinase-3β (GSK-3β), an endogenous inhibitor of Snail. The induction of Snail by *H. pylori* was regulated at multiple levels. These include increased transcription, inhibition of protein degradation, and enhancement of nuclear accumulation of Snail. Pre-treatment of AGS cells with *N*-acetylcysteine, a reactive oxygen species (ROS) scavenger, reversed the inactivation of GSK-3β signaling and attenuated the up-regulation of Snail by *H. pylori*. Intriguingly, when Snail was targeted by small interfering RNA (siRNA), the cell-cell interaction of AGS was intact regardless of the presence of *H. pylori*. These findings suggest that Snail expression induced by *H. pylori* and hummingbird phenotype as a consequence in human gastric cancer cells were mediated by GSK-3β signaling.

Keywords: *Helicobacter pylori*, ROS, GSK-3β, Snail, Hummingbird phenotype

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Introduction

H. pylori infects approximately half the world's population, possibly reaching up to 70% in developing countries and 20-30% in industrialized nations [1]. *H. pylori* infection has been discovered as a principle risk factor for gastric cancer. Although most infected individuals only experience chronic gastritis, a small subset of *H. pylori*-infected population develop gastric adenocarcinoma [2,3]. *H. pylori*, a gram-negative bacteria, triggers a sequence of alterations of the gastric mucosa starting with superficial gastritis, which can progress to chronic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia and finally gastric carcinoma [4]. Results from recent studies suggest that *H. pylori* infection is associated with an increase in epithelial-mesenchymal transition (EMT) in gastric cancer [5,6]. However, still the mechanism underlying *H. pylori*-induced EMT remains to be elucidated. Regarding EMT, epithelial cells acquire fibroblast-like properties and show reduced intercellular adhesion and increased motility, facilitating invasion and metastasis of tumor, which is known as one of hallmarks of cancer [7]. Normally, epithelial cells lined by basement membrane can abnormally grow and proliferate locally to

give rise to an adenoma. Further changes in epigenetic and genetic alterations result in carcinoma *in situ.* When *in situ* carcinoma cells undergo EMT, the basement membrane becomes fragmented and the cells can intravasate into lymph or blood vessel, allowing their passive transport to distant organs. At secondary location, solitary carcinoma cells can extravasate and either remain solitary (micrometastasis) or form a new carcinoma through a reversed process called mesenchymal-epithelial transition (MET), thereby accelerating tumor progression [8]. The transcription factor Snail has been considered as a key regulator of EMT [9,10]. In addition, over-expression of Snail was reported to be associated with lymph node metastasis and poor prognosis [11]. Based on these findings, I attempted to investigate whether *H. pylori* could induce Snail activation in human gastric cancer and to elucidate the underlying mechanism with focus on the plausible link between *H. pylori* infection and cell motility that can be regulated by Snail.

Materials and methods

Reagents

RPMI-1640 medium, fetal bovine serum, penicillin, streptomycin were products of GIBCO BRL (Grand Island, NY, USA). Sheep blood agar, Gaspak^{TM} and anaerobic jars were provided by BD Biosciences (Sparks, MD, USA). U0126 and LY294002 were purchased from TOCRIS (Ellisville, MO, USA). Carbobenzoxy-leucyl-leucyl-leucinal (MG132) was a product from Enzo Life Science (Farmingdale, NY, USA). Cyclohexamide (CHX), hydrogen peroxide (H_2O_2), N-acetylcysteine (NAC) and primary antibody against actin were products of Sigma-Aldrich Co. (St Louis, MO, USA). Primary antibodies for Snail, lamin B, phospho-p38, total p38 and p65 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phospho-Erk1/2, total Erk1, phospho-Akt, total Akt, phospho-GSK-3β, total GSK-3β, phospho-JNK and total JNK were purchased from Cell Signalling Technology (Beverly, MA, USA). Primary antibody for α-Tubulin was a product from Biogenex (Fremont, CA, USA). Horseradish peroxidise-conjugated secondary antibodies were obtained from Pierce Biotechnology (Rockford, IL, USA). Alexa 488

conjugated-IgG, TRIzol®, 2',7'-dichlorofluorescein diacetate (DCF-DA), propidium iodide (PI), SYBR®, Lipofectamine® RNAiMAX, and Steath™ RNAi negative control duplexes were provided by Invitrogen (Carlsbad, CA, USA). Human Snail-specific siRNA duplex (5'-GCGAGCUGCAGGACUCUAA-3') was purchased from Genolution Pharmaceuticals, Inc. (Seoul, Korea). Polyvinylidene difluoride membranes were supplied from Gelman laboratory (Ann Arbor, MI, USA). Protease inhibitor cocktail tablets were provided form Boehringer Mannheimm (Mannheimm, Germany). The ECL chemiluminescent detection kits were purchased from ELPIS-BIOTECH (Daejon, Korea) and GH Healthcare (Piscataway, NJ, USA). A protein assay dye (Bradford) reagent was supplied by Bio-Rad Laboratories (Hercules, CA, USA). The bicinchonic acid (BCA) protein assay reagent was obtained from Pierce Biotechnology (Rockford, IL, USA).

Cell culture

AGS cells and HCT116 cells were obtained from obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). AGS cells were cultured in RPMI-1640, and HCT116 cells were

maintained in DMEM medium supplemented with 10% v/v FBS and 100 units/mL penicillin and 100 µg/mL streptomycin at 37^0C in an incubator with humidified atmosphere of 95% O_2/5% CO_2.

Bacteria strain and growth condition

H. pylori (ATCC 43504, *cag* PAI, and *vac* A positive) was grown on 5% sheep blood agar plates and antibiotic supplements (Dents supplement) (Oxoid, Basingstoke, UK) at 37^0C under microaerophilic conditions generated by CampyPack plus in an atmosphere of 5% O_2, 10% CO_2, and 85% N_2 for 48 h.

In vitro H. pylori *infection model and drug treatment*

The cells were seeded in tissue culture plates for 24 h before infection. Sixteen h before infection, the medium was replaced by fresh RPMI 1640 without either antibiotic or serum. For the infection, bacteria were harvested in Brucella broth (Difco) containing 10% FBS and added to the host cells at a multiplicity of infection (MOI) of 100. U0126 (20 µM), LY294002 (20 µM), MG132 (20 µM) and N-acetylcysteine (NAC) (20 mM) were added to the cells 1 h before infection.

Scattering assay

Subconfluent cultures of AGS were infected with *H. pylori* in the presence or absence of expression of nonspecific siRNA or Snail siRNA for 10 h. Morphologic changes were assessed by light microscopy. Scattered colonies formation was verified by a typical change in morphology, characterized by a cell-cell dissociation and the acquisition of a migratory phenotype. Scattering activity was measured in the total number of scattered colonies from 50 colonies under a light microscopy.

Western blot analysis

After *H. pylori* infection, the cells were washed twice with phosphate-buffered saline (PBS) 1 x and snap-frozen in liquid nitrogen. Cells were then mixed with 1 x lysis buffer [100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA (pH 8.0), 1% Triton X-100, 5 mM dithiothreitol (DTT), 10% glycerol and protease inhibitor cocktail tablets] with 1% phenylmethylsulfonyl fluoride (PMSF) for 1 h on ice followed by centrifugation at 13 000*g* for 15 min at 4^0C. The protein concentration of the supernatant was measured by using the Bio-Rad protein assay dye (Bradford) reagent. The protein samples were solubilized with

sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample loading buffer and boiled for 8 min at 99^0C. The solubilized proteins (15-30 µg) were subjected to electrophoresis on 8% or 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane. The blots were then blocked with 5% fat-free dry milk (skim milk)-TBST (Tris-buffered saline containing 0.1% Tween-20) buffer for 1 h at room temperature. The blots were incubated with primary antibodies in 1% fat-free dry milk in TBST overnight. After three times washing with TBST, the blots were incubated with horseradish peroxidise-conjugated secondary antibodies in 1% fat-free dry milk-TBST for 1 h at room temperature. The blots were rinsed again three times with TBST and transferred proteins were incubated with Enhanced Peroxidase Detection Western blot Detection Kit (ELPIS-BIOTECH, Daejon, Korea) for 1 min or Amersham ECL Prime Western Blotting Detection Reagent (GH Healthcare, Piscataway, NJ, USA) according to manufacturer's instructions and visualized with the imagequant^{TM} LAS 4000 (Fujifilm Life Science, Stamford, USA).

Preparation of cytosolic and nuclear extracts

After *H. pylori* infection, cells were washed twice with ice-cold 1 x PBS, snap-frozen and then scraped in 1 ml 1 x PBS, followed by centrifugation at 13 000*g* for 10 min at 4^0C. Pellets were resuspended in hypotonic buffer A [10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.2 mM PMSF] for 15 min on ice. 10% Nonidet P-40 was then added to final concentration of 0.1% for 5 min. The mixture was then centrifuged at 13 000*g* for 5 min at 4^0C. Supernatant was collected as the cytosolic extract and stored at -70^0C. The pellets were washed twice with hypotonic buffer A and resuspended again in hypertonic buffer C [20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.9), 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM DTT and 0.2 mM PMSF] for 1 h on ice and centrifuged at 13 000*g* for 15 min at 4^0C. The supernatant containing nuclear proteins was collected and stored at -70^0C. The protein concentrations of cytosolic and nuclear extracts were determined using the BCA protein assay reagent.

Reverse transcriptase-PCR (RT-PCR)

Total RNA was isolated from AGS cells using TRIzol® and then used for the complementary DNA synthesis using random primers. Reverse transcriptase-PCR was performed following standard procedures. PCR conditions for *Snail* and the house keeping gene *GAPDH* were as follows: 25 cycles of 95^0C for 20 s; 60^0C for 30 s and 72^0C for 40 s. The primer pairs of the expected products were as follows (forward and reverse, respectively): Snail, 5'-CCTGCTGGCAGCCATCCCAC-3' and 5'-GGCAGCGTGTGGCTTCGGAT-3' and *GAPDH*, 5'-ACCCAGAAGACTGTGGATGG-3' and 5'-TCTAGACGGCAGGTCAGGTC-3'. Amplified products were resolved by 1.5% agarose gel electrophoresis, stained with SYBR® and visualized with the imagequant™ LAS 4000.

Snail-siRNA transient transfection

AGS cells were transfected with *Snail*-siRNA or control siRNA using the transfection reagent Lipofectamine RNAiMAX for 24 h following the manufacturer's instructions. Transfected cells were then treated with *H. pylori* for the indicated times and then harvested for the next experiments.

Immunocytochemical analysis of Snail

To investigate localization of Snail, immunocytochemistry was conducted. AGS cells were infected with *H. pylori* for the indicated intervals. After fixation with 4% paraformaldehyde for 20 min at room temperature, samples were permeabilized with 0.2% Triton X-100 and then blocked with 5% bovine serum albumin in PBST (PBS containing 0.1% Tween-20) for 2 h at room temperature. Samples were then stained with primary antibody specific for Snail overnight at 4^0C, followed by incubation with fluorescein isothiocyanate-goat anti-rabbit IgG secondary antibody for 1 h at room temperature. Nuclear-staining was performed with propidium iodide (PI) for 10 min at room temperature. Images were assessed under a fluorescent microscopy (Leica, Wetzlar, Germany).

Measurement of ROS accumulation

Accumulation of ROS in AGS cells infected with *H. pylori* was monitored using the fluorescence-generating probe DCF-DA. Treated cells were rinsed with PBS and loaded with 10 μM DCF-DA. After 30 min incubation at 37^0C, cells were examined under a fluorescent microscopy set at 488 nm for excitation and 530 nm for emission.

Human gastric mucosa biopsy

Gastric biopsy specimens were obtained from patients by esophagogastroduodenal (EGD) endoscopy in Seoul National University Bundang Hospital (Seoul, Korea).

Statistical analysis

Data from three independent experiments at least were expressed as the mean ± s.e.m. The statistical significance of differences between two groups was evaluated using Student's *t* test. Analysis was performed using Graphpad Prism (Version 6). Statistical significance was accepted at $P \leq 0.05$, unless otherwise indicated.

Results

H. pylori *disrupts cell-cell interaction and up-regulates Snail expression in gastric epithelial AGS cells*

The morphology and behavior of AGS cells treated with *H. pylori* were assessed by optical microscopy. Upon exposure to *H. pylori*, cells were dispersed and exhibited a spindle-like appearance (Figure 1A). Moreover, the number of scattered colonies, reflecting cell motility and invasive ability, was enhanced compared with non-infected controls (Figure 1B). Snail has been best known to play a key role in induction of EMT, a process converting epithelial cells into mesenchymal cells with changes in cell morphology and acquisition of migratory properties [12]. In *H. pylori*-treated AGS cells, a time-dependent increase in Snail protein levels was observed (Figure 1C). To further investigate the role of Snail in EMT of AGS cells, we utilized siRNA specifically targeting Snail. The effectiveness of this siRNA to knock down Snail expression was confirmed by RT-PCR whereas nonspecific siRNA had no effect (Figure 1D). As illustrated in Figure 1E, the hummingbird phenotype as well as intercellular separation

caused by *H. pylori* infection was blocked by introduction of Snail siRNA (Figure 1E).

Erk regulates H. pylori*-induced Snail expression*

Since activation of mitogen-activated protein kinase (MAPKs) and downstream effectors such as NF-κB has been reported to be involved in EMT [13], we attempted to investigate whether these upstream kinases regulate up-regulation of Snail expression in *H. pylori* -infected cells. We initially examined whether *H. pylori* could induce phosphorylation of MAPKs. It was revealed that, *H. pylori* induced phosphorylation of Erk much earlier (Figure 2A, 2B) than JNK or p38 (Figure 3), suggesting the plausible involvement of Erk in induction of Snail expression. To confirm the role of Erk in regulation of Snail expression by *H. pylori*, U0126 (an MEK inhibitor) was applied. The pharmacologic inhibition of MEK-Erk signaling by U0126 abolished phosphorylation of Erk induced by *H. pylori* (Figure 2C). In addition, pre-treatment with U0126 resulted in an abrogation of *H. pylori*-induced Snail expression (Figure 2D, 2E), suggesting that *H. pylori* -induced Snail expression is mediated, at least in part, by Erk. We then checked the expression level of NF-κB, a common downstream effector

of MAPKs [14-16], not only in the whole-cell lysate but also in cytosolic and nuclear fractions. However, we could not observe any changes in the level of p65, a fuctionally active subunit of NF-κB, following *H. pylori* treatment (Figure 4).

***PI3K/Akt/GSK-3β is involved in induction of Snail expression by* H. pylori**

GSK-3β, a kinase located downstream of PI3K/Akt pathway, has been known as an endogenous inhibitor of Snail by targeting this protein for proteasomal degradation when GSK-3β maintains an active state in a dephosphorylated form [17-19]. Notably, GSK-3β was inactivated by *H. pylori* which appears to be associated with activation of PI3K/Akt signaling. This may result in phosphorylation of GSK-3β at Ser9, keeping it in an inactivate form, as shown by an increase in p-Akt and p-GSK-3β (Ser9) (Figure 5A, 5B, 5C). To confirm that the up-regulation of Snail by *H. pylori* is mediated through supression of GSK-3β activity, we treated AGS cells with LY294002, a PI3K inhibitor, prior to *H. pylori* treatment and then expression of p-Akt, p-GSK-3β, and Snail was examined by Western blot analysis. We found that LY294002 inhibited the effect of *H. pylori* on the phosphorylation

of GSK-3β as well as Akt (Figure 5D). In addition, LY294002 also blocked induction of Snail expression by *H. pylori* (Figure 5E, 5F). According to some previous studies, Erk could act upstream of GSK-3β [20,21]. However, we did not see any changes in phosphorylation of GSK-3β by *H. pylori* in the presence of MEK inhibitor (Figure 6).

H. pylori *increases mRNA synthesis, protein stability and nuclear translocation of Snail*

In another experiments, RT-PCR was performed to check the mRNA level of Snail. *H. pylori* induced an increase in the Snail mRNA level at 3 h (Figure 7A). Consistent with inhibition of *H. pylori*-induced expression of Snail by U0126 treatment, the levels of *Snail* mRNA were also markedly reduced. However, LY294002 failed to suppress (Figure 7C). These data suggest that there must be another mechanism alternative to transcriptional up-regulation of Snail expression. Snail has been discovered as a highly unstable protein with a half-life of 25 minutes [22]. In agreement with this notion, Snail protein is barely detectable in untreated AGS cells. Thus, we conducted a chase experiment by use of CHX, a protein synthesis inhibitor, to more precisely assess the stability of Snail protein. AGS were treated with *H.*

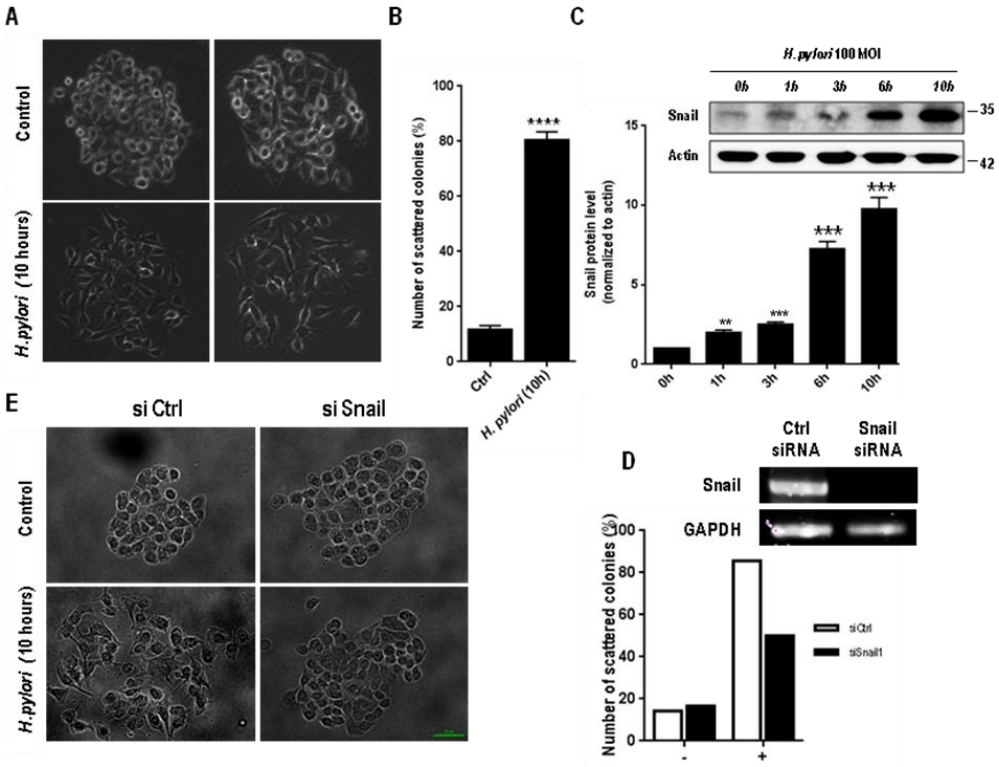
pylori for 6 h to induce Snail expression and then exposed to DMSO or CHX. As shown in Figure 7D and E, CHX treatment did alter the Snail protein expression profile.

Snail function could be affected by its subcellular localization. When it is located in cytosol, it is phosphorylated and subsequently targeted for proteasomal degradation [23]. We noticed a prominent nuclear accumulation of Snail following *H. pylori* infection (Figure 7F and Figure 8). The increased nuclear localization of Snail was further verified byWestern blot analysis (Figure 7G).

The ROS scavenger attenuates H. pylori*-induced Snail expression by inhibiting p-Erk1/2 and p-GSK-3β*

An accumulation of intracellular ROS alters gene expression [24,25] and stimulates cell invasiveness [26,27]. The generation of intracellular ROS has been reported to be enhanced in gastric epithelial cells as a consequence of *H. pylori* infection [28,29]. Thus, we speculated that *H. pylori* might utilize ROS to mediate Snail expression. We initially measured the generation of ROS after *H. pylori* exposure using the oxidant-sensitive fluorescent probe DCF-DA. The enhancement of ROS production by *H. pylori* was not only detected in

AGS cells (Figure 9B) but also specimens from *H. pylori*-infected patients gastric biopsy (Figure 9A) as compared with normal individuals. The antioxidant NAC abolished not only ROS accumulation (Figure 9B, bottom panels) but also Snail expression (Figure 9C) in *H. pylori*-treated cells. Moreover, we also noticed that pre-incubation with NAC blocked the activation of Erk and inactivation of GSK-3β caused by *H. pylori* through downregulation of phosphorylation of these proteins (Figure 9D). These data indicate that *H. pylori*-induced Snail expression in AGS cells is mediated, at least in part, via increased ROS production.


A
Control
H.pylori (10 hours)
B
Number of scattered colonies (%)
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Ctrl
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C
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0h
1h
3h
6h
10h
Snail
Actin
35
42
Snail protein level (normalized to actin)
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E
si Ctrl
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Control
H.pylori (10 hours)
D
Ctrl siRNA
Snail siRNA
Snail
GAPDH
Number of scattered colonies (%)
siCtrl
siSnail1

Figure 1. Disrupted cell-cell interaction and up-regulated Snail expression in *H. pylori*–treated human gastric cancer AGS cells. (**A**) AGS cells co-incubated with *H. pylori* for 10 h were subjected to the cell scatter assay. Representative images show a spindle-like shape as well as destruction of direct interaction between cells. (**B**) The number of scattered colonies was scored. (**C**) AGS cells were treated with *H. pylori* for the indicated times, followed by immunoblotting with Snail antibody. Actin was included to ensure equal protein loading. Immunoblots were quantified by densitometry. (**D**) AGS cells were transfected with either siRNA of Snail or control siRNA for 24 h. Total RNA was then extracted and analyzed by reversed transcriptase-PCR with Snail sequence-specific primers, showing complete silence of *Snail* by Snail siRNA. *GAPDH* was used as an internal control. (**E**) AGS cells were treated with *H. pylori* for 10 h in the cells transfected with control siRNA or Snail siRNA. Bright field microscopy reveals that the *H. pylori*-caused hummingbird phenotype and intercellular separation are abrogated by siRNA knock down of Snail. All results are presented as mean ± s.e.m; **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. Scale bar: 50 μm (A, E).

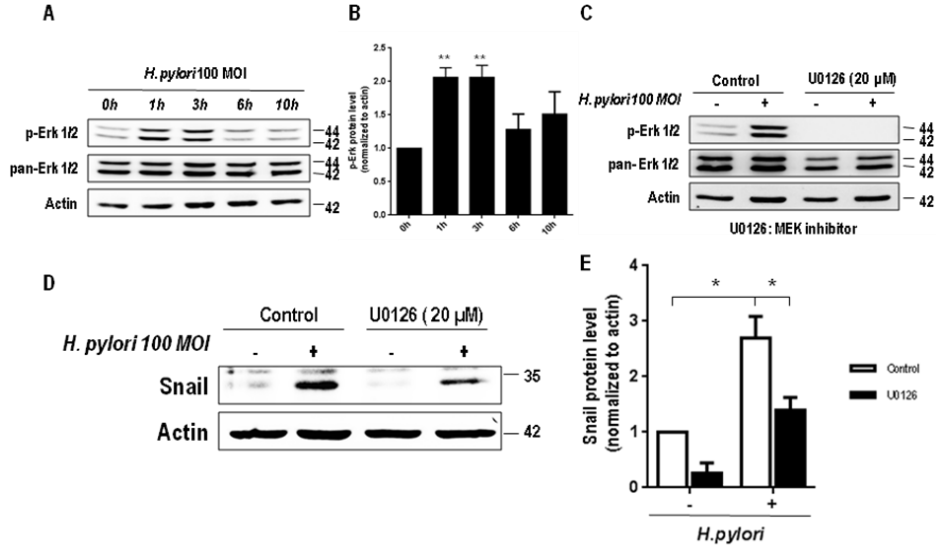

Figure 2. Role of Erk in *H. pylori*-induced Snail expression. (**A**) Whole-cell lysates from AGS cells treated with *H. pylori* were subjectd to Western blot analysis. (**B**) Densitometric quantification of p-Erk1/2 was shown. Data represent mean ± s.e.m of three independent assays. (**C**-**E**) Cells were pre-treated with 20 μM of U0126, an MEK inhibitor, for 1 h prior to incubation with *H. pylori* for additional 3 h. (**C**) Levels of p-Erk1/2 were determined by Western blot analysis, showing complete inhibition of Erk1/2 phosphorylation by U0126. (**D**) Abrogation of *H. pylori*-induced Snail expression by U0126. (**E**) Densitometric quantification of Snail. Data represent mean ± s.e.m of three independent assays. *$P \leq 0.05$, **$P \leq 0.01$.

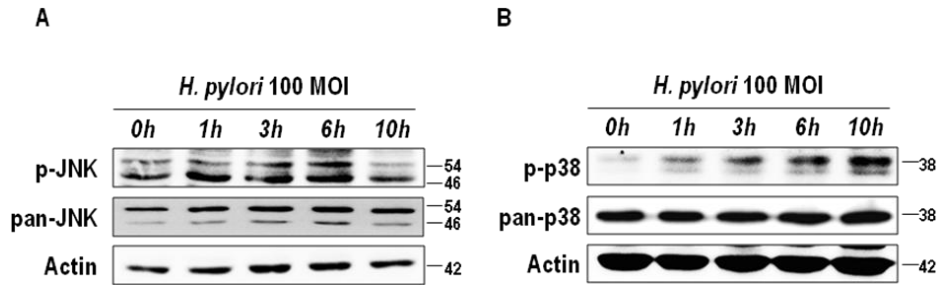

Figure 3. **Effect of *H. pylori* treatment on phosphorylation of JNK (A) and p38 (B) in AGS cells.** Western blot analysis was done for p-JNK and p-p38 in AGS treated with *H. pylori*.

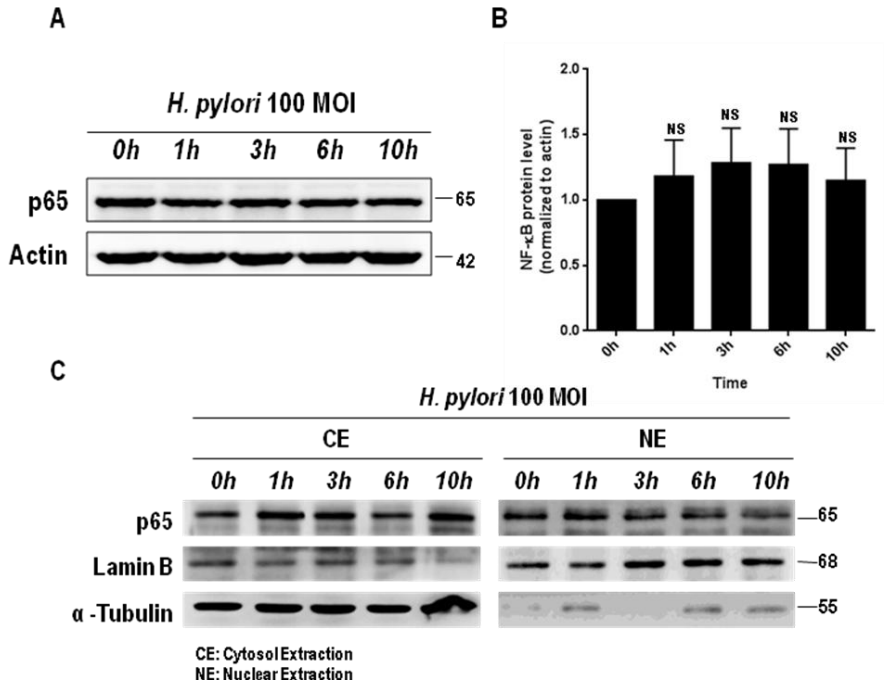

Figure 4. **Effects of *H. pylori* treatment on expression and nuclear localization of p65 subunit of NF-κB**. Either whole cell-lysates (**A**) or cytosolic and nuclear extracts (**C**) from AGS cells co-incubated with *H. pylori* for indicated times were subjected to immunoblotting with p65-specific antibody. (**A**) The p65 protein level in whole-cell lysate was not changed by *H. pylori* treatment. (**B**) Density of p65 bands was quantified and shown. Data represent the mean ± s.e.m of three independent assays. (**C**) p65 protein levels in cytosolic (CE) or nuclear (NE) are shown. Blots are representative of three independent experiments.

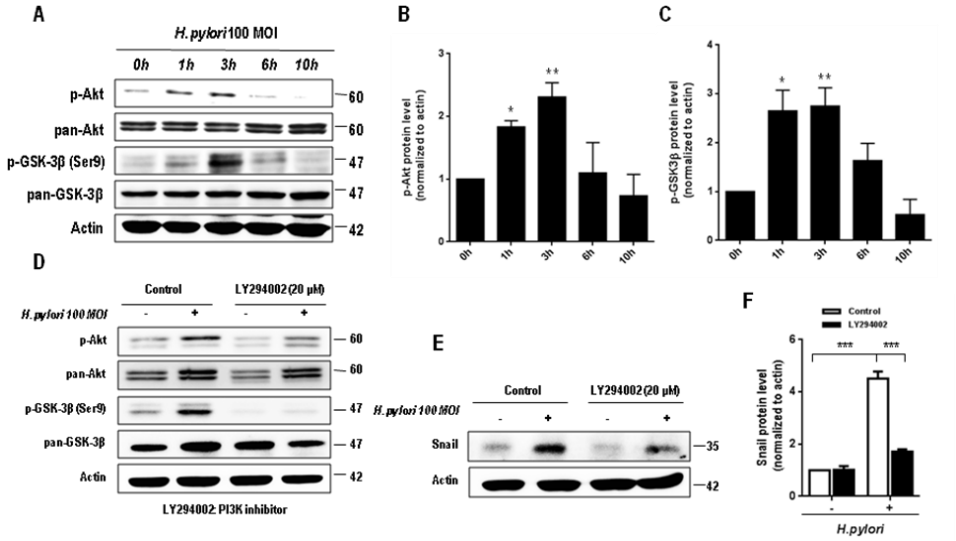

Figure 5. Involvement of the PI3K/Akt/GSK-3β axis in the induction of Snail expression by *H. pylori*. AGS cells were treated with *H. pylori* and whole-cell lysates were prepared for Western blot analysis of p-Akt and p-GSK-3β. (**B-C**) Densitometric quantification of p-Akt and p-GSK-3β is shown. Data represent mean ± s.e.m of three independent assays. (**D-F**) Cells were pre-incubated with 20 μM of LY294002, a PI3K inhibitor, for 1 h prior to co-incubation with *H. pylori* for additional 3 h and then collected for Western blot analysis of p-Akt, p-GSK-3β and Snail. (**D**) Complete inhibition of phosphorylation of Akt as well as GSK3-β by LY294002. (**E**) Inhibitory effects of LY294002 on *H. pylori* -induced Snail expression. (**F**) Density of Snail bands was quantified. Data represent mean ± s.e.m of three independent assays. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

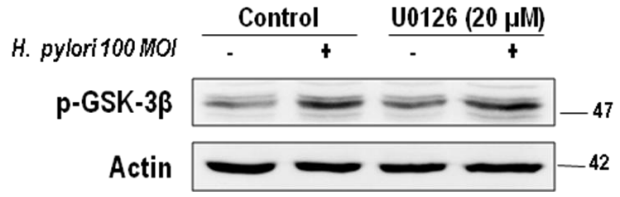

Figure 6. Effects of Erk inhibition on *H. pylori*-induced GSK-3β phosphorylation. AGS cells were treated with U0126 (20 μM) for 1 h prior to *H. pylori* exposure for additional 3 h. Western blotting analysis of Snail was performed, showing no effect of U0126 on *H. pylori*-induced phosphorylation of GSK-3β.

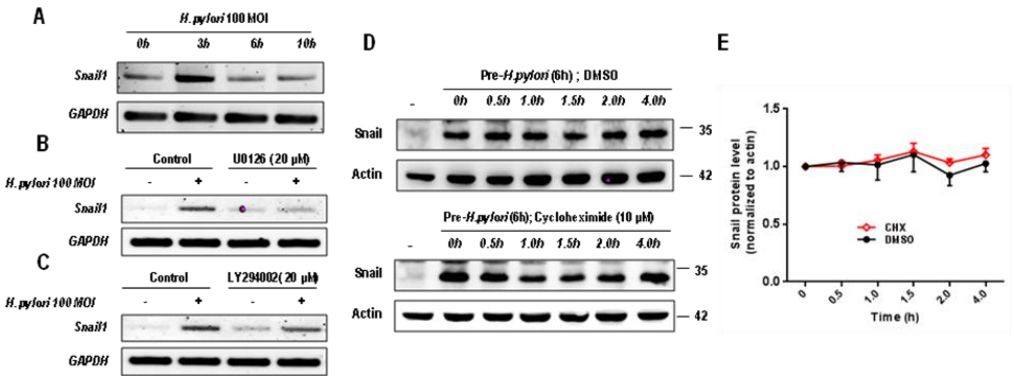

Figure 7. Increased mRNA synthesis, protein stability and nuclear translocation of Snail in AGS cells treated with *H. pylori.* (**A**) Total RNA from AGS cells exposed to *H. pylori* was extracted and then reverse transcribed followed by PCR to determine the levels of *Snail* messenger RNA (mRNA). (**B-C**) Cells were pre-treated with U0126 (20 μM) or LY294002 (20 μM) for 1 h, prior to incubation with *H. pylori* for additional 3 h. Total RNA was prepared for RT-PCR with specific primers for Snail. Inhibition of *H. pylori*-induced *Snail* mRNA expression by U0126 (**B**) but not by LY294002 (**C**). (**D-E**) AGS cells were pre-treated with *H. pylori* for 6 h before exposure to DMSO or CHX (10 μM), and Snail expression was measured. All data are representative of three independent assays.

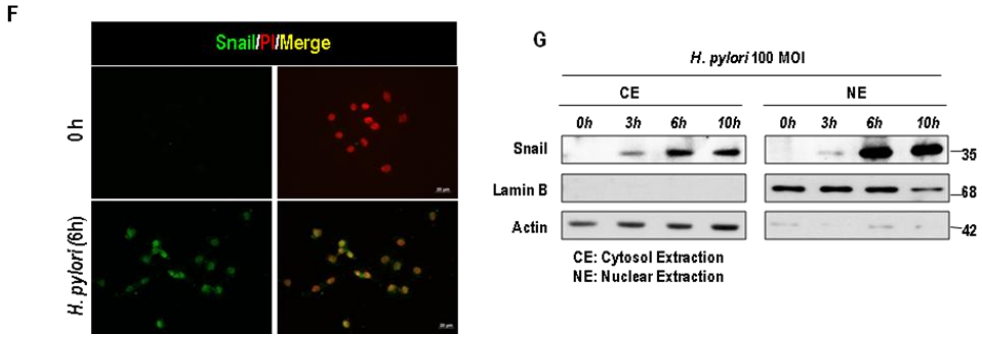

Figure 7. Increased mRNA synthesis, protein stability and nuclear translocation of Snail in AGS cells treated with *H. pylori.* (**F**) Nuclear accumulation of Snail in AGS cells upon *H. pylori* infection for 6 h was observed by fluorescent microscopy after immunofluorescence staining. Scale bar: 50 μm. (**G**) Nuclear translocation of Snail was verified by immunoblot analysis. Actin and lamin B were included as loading controls for cytosolic and nuclear fractions, respectively. Data are representative of three independent assays.

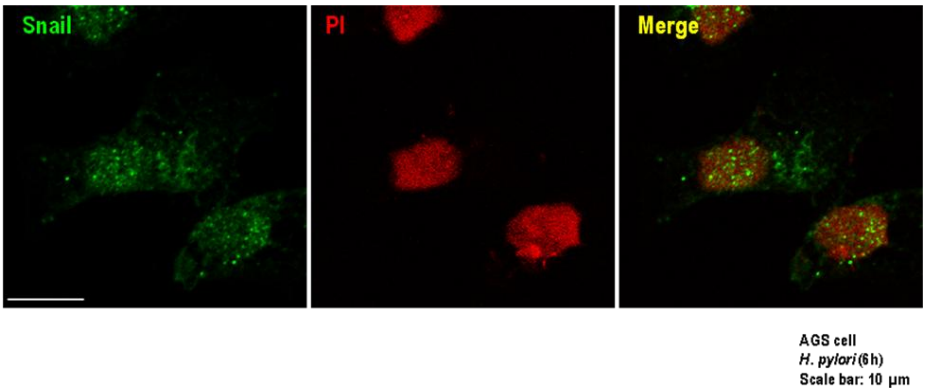

Figure 8. Intracellular localization of Snail. Blow-up of immunocytochemical result of Figure 7F demonstrates that *H. pylori*-induced *H.pylori*-induced Snail is predominantly localized in nucleus.

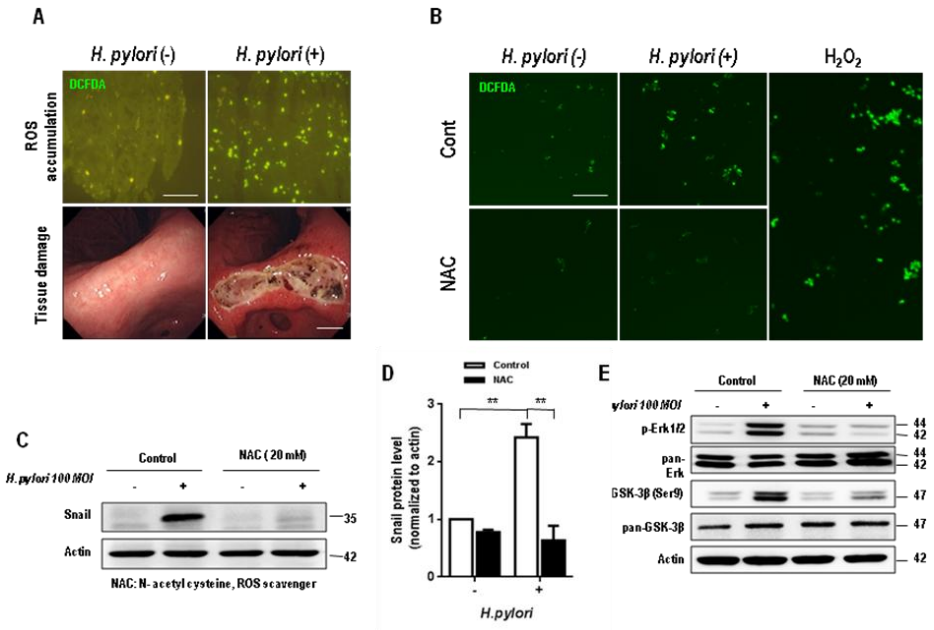

Figure 9. Possible involvement of ROS in *H. pylori*-induced Snail expression by targeting Erk1/2 and GSK-3β. (**A**) Human gastric tissue specimens were stained with DCF-DA and examined under fluorescent microscopy. Intracellular ROS accumulation was associated with severe tissue damage in a *H. pylori*-positive sample compared to *H. pylori*-negative one. (**B-D**) AGS cells were treated with NAC for 1 h prior to exposure to *H. pylori* for 30 min (**B**) or 3 h (**C-D**). (**B**) H_2O_2 was employed as a positive control. Cells were then washed and incubated with DCF-DA, followed by fluorescent light microscopy. (**C-D**) *H. pylori*-induced expression of Snail was abolished by NAC. (**E**) Phosphorylation of Erk1/2 and GSK-3β triggered by *H. pylori* was almost completely abolished by NAC treatment. Data represent mean ± s.e.m of three independent assays. **$P \leq 0.01$.

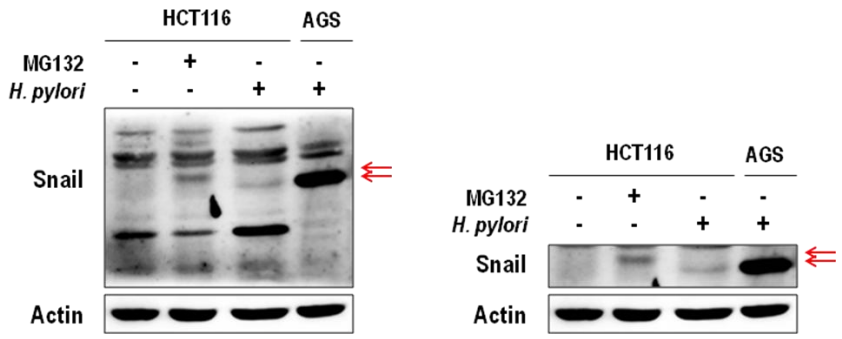

Figure 10. ***H.pylori*** **induced Snail in a non-phosphorylated form.** HCT116 cells were treated with MG132 (20 μM), a proteasome inhibitor, or *H. pylori* for 6 h. AGS were incubated with *H. pylori* for 6 h. Whole-cell lysates were subjected to Western blot analysis with Snail-specific antibody. Snail in *H. pylori*–treated cells migrated faster on SDS-PAGE gel than that in MG132 treated cells.

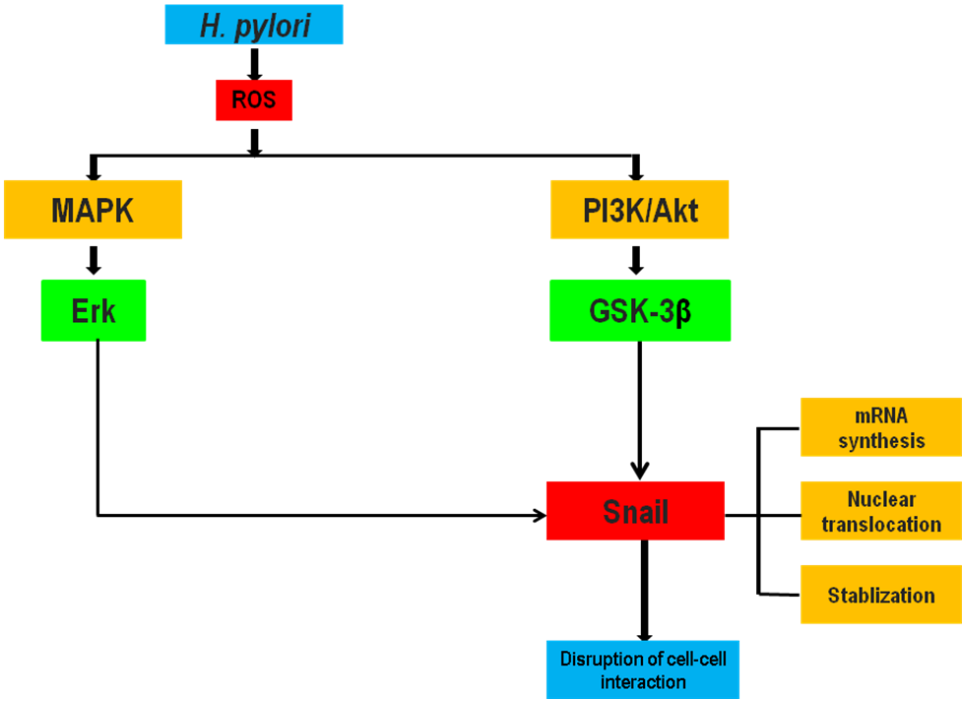

Figure 11. **A proposed mechanism underlying the induction of Snail by *H. pylori***. The study provides an underlying mechanism for induction of Snail and the hummingbird phenotype in AGS cells treated with *H. pylori*. The crucial role of ROS as a feasible link between *H. pylori* and this up-regulation of Snail is addressed.

Discussion

Expression of the transcription factor Snail is a critical determinant in the acquisition of a mesenchymal phenotype characterized by the spindle-like shape and migratory properties that contribute to acquisition of invasiveness of epithelial tumor cells [30]. In this study, we showed that *H. pylori*, a crucial factor implicated in gastritis and stomach cancer, upregulated expression of this transcription factor and resulted in gain of hummingbird phenotype as well as an increase in cell motility in human gastric epithelial AGS cells. Consistent with this observation, knockdown of Snail expression abrogated these effects of *H. pylori*.

Our study provides an insight into the mechanism underlying regulation of Snail by *H. pylori*. Firstly, this bacterium activated Ekr and PI3K/Akt/GSK-3β signalings which lead to an up-regulation of Snail expression. In other studies, it was shown that a blockage of the MEK/Erk pathway effectively inhibited the PI3K/Akt/GSK-3β signaling [31]. However, we did not observe any effect of an Erk inhibitor on activation of the PI3K/Akt/GSK-3β pathway, indicating that they worked independently of each other to mediate expression of

Snail. Once Erk was phosphoryated by *H. pylori*, it is likely to be involved in transcriptional regulation of the Snail gene in early time of infection. By contrast, GSK-3β appears to act differently. Other investigators have reported that Snail activity is regulated by its subcellular localization. When it is localized in cytosol, Snail is targeted to phosphorylation by upstream signaling kinases and followed by ubiquitin-proteasomal degradation [32-34]. Notably, Snail has been also reported to be a highly unstable protein with a half-life of 25 min [22], indicative of participation of several factors in maintaining Snail stabilization. Similarly, in current study, we noticed that, GSK-3β did not affect an mRNA level but only protein level of Snail. It was also supported by a decrease in the turnover along with an increase in nuclear accumulation of Snail in AGS cells exposed to *H. pylori*, predicting an intact state of Snail due to this exposure. In addition, this prediction can be partially clarified when we compared the state of Snail induced by *H. pylori* to that in the cells treated with MG132, a well-known proteasome inhibitor. In the presence of MG132, Snail is maintained in phosphorylated and ubiquitinated forms. As a result, the movement of Snail on SDS-PAGE gel is delayed [35]. However, Snail in *H. pylori* infected samples did migrate faster than that from MG132

treated samples, suggesting the existence of either non-phosphorylated or non-ubiquitinated form of Snail induced by *H. pylori* infection to prevent itself from ubiquitin-proteasomal degradation (Figure 10).

Overexpression of Snail has been associated with invasion, metastasis and poor prognosis, particularly, in gastric cancer [12,36]. Once Snail is inactivated, invasive and metastatic ability of cancer cells can be inhibited [37,38]. Notably, intracellular ROS accumulation in *H. pylori*-positive human gastric mucosa biopsy was higher than that in *H. pylori*-negative samples. Additionally, *H. pylori*-induced Snail expression can be almost completely blocked by application of the ROS-quenching reagent, NAC. ROS could be a plausible link between *H. pylori*–induced EMT and activation of Erk and PI3K/Akt/GSK-3β pathways. Through its ability to scavenge ROS, NAC inhibited phosphorylation of Erk and GSK-3β, leading to reduction of Snail expression.

In summary, our study reveals that, *H. pylori* up-regulates expression of Snail and causes a hummingbird via multiple mechanisms including the increase in Snail transcription, the prevention of Snail protein degradation, and the enhancement of Snail nuclear localization

which partly involve activation of Erk and more notably inactivation of GSK-3β.

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