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# *Helicobacter pylori* CagA Inhibits the Expression of Runx3 Via Src/MEK/ERK and p38 MAPK Pathways in Gastric Epithelial Cell

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## ABSTRACT

Infection with CagA-positive *Helicobacter pylori* is the strongest risk factor for gastric carcinoma. Upon delivery into gastric epithelial cells, CagA disturbs cellular functions by physically interacting with and deregulating intracellular signaling molecules via both tyrosine phosphorylation-dependent and -independent mechanisms. Runx3 was suggested to be a tumor suppressor and closely associated with tumorigenesis and progression of gastric cancer. The aim of our study is to verify the effect of *H. pylori* virulence factor CagA on Runx3 expression level and investigate the corresponding molecular mechanisms and signaling pathways influencing Runx3 expression. Human gastric epithelial immortalized GES-1 cells were transfected with CagA-expression vector or control vector with FuGENE HD transfection reagent. Runx3 expression levels were determined by QRT-PCR and immunoblotting. Then we constructed a 1,150 bp Runx3 promoter luciferase reporter plasmid, $pGL_3$-1150 bp, which was co-transfected into GES-1 cell with CagA-expression vector or control vector. Luciferase reporter assay was used to determine the effects of CagA on the 1,150 bp promoter activity of Runx3. Signal inhibitors were used to detect the signal pathway(s) through which CagA affects Runx3. Our results showed that CagA can reduce the expression level of Runx3 at both mRNA and protein levels significantly. Importantly, the 1,150 bp Runx3 promoter activity was decreased in cells transfected with CagA-expression vector comparing with cells transfected with control vector. And this inhibition is dependent on the phosphorylation of CagA. Signal pathways Src/MEK/ERK and p38 MAPK are involved in this regulation. Our findings provide new insights for understanding the mechanism of *H. pylori* carcinogenesis. J. Cell. Biochem. 113: 1080–1086, 2012. 

**KEY WORDS:** CagA; Runx3; REGULATION

*Helicobacter pylori* (*H. pylori*) colonizes the human stomach of at least half of the world's population and causes many digestive diseases, such as chronic gastritis, gastroduodenal ulcer [Dooley et al., 1989] as well as gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue lymphoma [Parsonnet et al., 1991; Covacci et al., 1999; Cover and Blaser, 1999]. CagA is one of the most important virulence factors secreted by *H. pylori*. Infection by cagA-positive *H. pylori* strains is associated with an increased risk for gastric cancer compared with infection by cagA-negative strains [Blaser et al., 1995; Eck et al., 1997; Covacci et al., 1999], implying an important role for CagA in *H. pylori*-associated gastric diseases. After *cagA*-positive *H. pylori* adheres to gastric epithelial cells, the CagA protein can be translocated from the bacteria into the host cell via a type IV secretion system (TFSS) which is encoded by the gene cag-PAI [Asahi et al., 2000; Stein et al., 2000]. Within gastric epithelial cells, CagA involves in many intracellular signal pathways and causes a series of cellular events. The C terminus of CagA can be tyrosine phosphorylated at its Glu-Pro-Ile-Tyr-Ala (EPIYA) motif by Src kinase and c-Abl kinase [Odenbreit et al., 2000; Selbach et al., 2002; Tsutsumi et al., 2003]. Phosphorylated CagA

Zhifang Liu and Xia Xu contributed equally to this work.

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interacts with the Src-homology 2 (SH2) domains of the protein tyrosine phosphatase SHP-2, thus stimulating its phosphatase activity [Higashi et al., 2002]. Activated SHP-2 is able to induce Ras/Raf/MEK/ERK pathway. On the other hand, CagA protein, independent of the tyrosine phosphorylation, can also interact with other signaling molecules, such as growth factor receptor binding protein 2 (Grb2) [Mimuro et al., 2002; Rieder et al., 2005], hepatocyte growth factor (HGF)/scatter factor receptor c-Met [Churin et al., 2003], phospholipase C-γ (PLC-γ) and deregulate Gsk-3β/β-catenin pathway. Deregulation of these pathways by CagA may result in the inflammation and the change of cell morphology, cell motility, cell scattering, cell proliferation, and intercellular tight junctions.

Runx3 was suggested to be a tumor suppressor gene in gastric cancer and closely associated with tumorigenesis and progression of gastric cancer [Li et al., 2002]. Runx3-deficient mice exhibit hyperplasias in gastric mucosa due to reduced apoptosis and stimulated proliferation of gastric epithelial cells. Runx3−/− gastric epithelial cells are less sensitive to the proapoptotic and growth inhibitory effects of transforming growth factor-β (TGF-β). Furthermore, Runx3 is inactivated in most of the gastric malignant cell lines, such as AGS, MKN28, MKN45, KATOIII, as well as 40% of early stage and in nearly 90% of advanced stage gastric carcinomas by hemizygous deletion and hypermethylation of its promoter. Restoration of Runx3 expression suppressed the growth and metastasis of human gastric cancer cells in ectopic and orthotopic animal models [Wei et al., 2005]. Study shows that Runx3 can interact with FoxO3a in gastric cancer cell lines to activate *Bim* and undergo TGF-β induced apoptosis [Yamamura et al., 2006; Yano et al., 2006].

Our aim in this article is to study the regulatory effects of *H. pylori* CagA on the expression of human *Runx3* gene in gastric epithelial immortalized cell line GES-1 and investigate the regulation mechanism and signal pathway(s).

## METHODS

### PLASMID

Wild-type (WT) cagA/pcDNA3.1(+) plasmid (WT-cagA) and a phosphorylation-resistant (PR) plasmid derivative (PR-cagA) were kindly provided by Zhu Yongliang (Zhejiang University, China). These two plasmids were characterized as described previously [Zhu et al., 2004, 2005]. The pcDNA3.1(+) mammalian expression vector was purchased from Invitrogen.

### CELL CULTURE

GES-1 cell line was conserved by our laboratory. The cells were routinely cultured in RPMI 1640 medium (Gibco Life Technologies) supplemented with 10% fetal bovine serum (Gibco Life Technologies), 100 U/ml penicillin and 100 U/ml streptomycin in air with 5% $CO_2$ at 37°C.

### CELL TRANSFECTION

FuGENE HD Transfection Reagent (Roche Applied Science) was used to transfect plasmid WT-cagA, PR-cagA, or pcDNA3.1 control vector into GES-1 cells. All transfection procedures were performed according to the protocol provided by the manufacturer and all experiments were repeated three times. After the transfection for 24 or 48 h, cells were harvested and extracted for RNA or protein. In order to investigate the signal pathways, we used the following reagents: BAY11-7082 (5 μM), PP1 (20 μM), PD98059 (50 μM), and SB203580 (10 μM). We used one of these reagents to pre-incubate cells for 30 min, and then transfected plasmids into cells for 24 or 48 h. Then RNA or protein was extracted from the cells. Also we transfected GES-1 with plasmid WT-cagA or pcDNA3.1 control vector. At 36 h after transfection, cells were treated with or not with MG-132 (10 μM) for 6 h. Then protein was extracted from the cells.

### RNA EXTRACTION, REVERSE TRANSCRIPTION AND QRT-PCR

Total RNA was extracted from treated cells with the TRIzol reagent (Invitrogen) following the manufacturer's instructions. cDNA was synthesized using random primers (N6) and M-MLV reverse transcriptase (MBI). QRT-PCR was carried out in an ABI7700 sequence detector (Applied Biosystems, Foster City, CA) with SYBR Green kit (Applied Biosystems). Levels of target mRNA were calculated based on the CT values and normalization of human β-actin expression.

### WESTERN BLOT

Protein concentrations were measured by BCA reagent kit (Merck). Protein samples were separated by SDS–PAGE, and then the proteins were transferred onto a nitrocellulose membrane which was blocked immediately with 5% nonfat milk in phosphate-buffered saline for 1 h at room temperature. After being blocked and washed, the membrane was incubated with human specific anti-CagA antibody (Santa Cruz Biotechnology, Inc., CA), anti-phosphorylated tyrosine (pY99, Santa Cruz Biotechnology, Inc.), or anti-Runx3 antibody (Abcam) at 4°C for 16 h, followed by incubation with peroxidase-labeled second antibody for 1 h at room temperature, then immunoreactive bands were visualized by enhanced chemiluminescence (ECL) (Santa Cruz Biotechnology, Inc.). β-tubulin or β-actin (BD Biosciences) was used to normalize the quantity of the protein on the blot.

### LUCIFERASE REPORTER ASSAY

Promoter sequence of *Runx3* (+28 to −1,122 bp) was amplified from the genomic DNA of white blood cell by PCR. The PCR primers were as follows: sense primer: 5′-GGGGGTACCGCCTGCAAGATCCT-GAAAC-3′ and antisense primer: 5′-GGG CTCG AGGGGAATACG-CATAACAGCG-3′. PCR conditions consisted of initial denaturation at 94°C for 3 min and 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 45 s, and primer extension at 72°C for 90 s. The final primer extension was performed at 72°C for 10 min. The promoter sequence was then cloned into pGL3-basic vector to construct the reporter vector (named as pGL3-1150). GES-1 cells were plated into 24-well plates and incubated to 90% confluence. WT-cagA, PR-CagA, and pcDNA3.1 was co-transfected into GES-1 cells with pGL3-1150 using the FuGENE HD Transfection Reagent (Roche Applied Science). pRL-TK was used as transfection efficiency control. In order to investigate the signal pathways, we used one of the following reagents: BAY11-7082 (5 μM), PP1 (20 μM), PD98059 (50 μM), and SB203580 (10 μM) to pre-incubate cells for 30 min, and then we transfected plasmids into the cells. Luciferase reporter

assays were performed using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instructions. Each experiment was performed in triplicates and repeated twice.

### STATISTICAL ANALYSIS OF DATA

The Statistical Package for the Social Sciences (SPSS) was used in the statistical analysis, and a Student's *t*-test was used to determine statistical significance. $P < 0.05$ were considered statistically significant.

## RESULTS

### Runx3 EXPRESSION WAS INHIBITED BY *H. PYLORI* WT-CagA IN GES-1 CELLS

In order to evaluate the effect of *H. pylori* CagA on Runx3 expression level, GES-1 cell were transfected with WT-CagA or pcDNA3.1, respectively. We used Western blot to detect the expression of CagA protein and the phosphorylation of WT-CagA in the GES-1 cell at 48 h after the WT-cagA plasmid transfection. The result illustrated in Figure 1A showed that in GES-1 cell, CagA has a very high expression level after transfection. Also we can detect a clear band with the antibody PY99 which was used to detect the phosphorylated CagA [Zhu et al., 2004]. This result showed that the WT-CagA can be phosphorylated in GES-1 cell after transfection. Then we used QRT-PCR and Western blot to estimate the expression of Runx3 at 24 and 48 h after transfection. The results showed that in GES-1 cell, WT-CagA can inhibit Runx3 expression both at RNA and protein level significantly (Fig. 1B,C). Then we used the luciferase reporter assay to determine whether WT-CagA can affect the promoter activity of Runx3. Figure 1D showed that the activity of the *Runx3* promoter was inhibited by WT-CagA significantly, which is consistent with the results of QRT-PCR. From the results, we concluded that the inhibitory effects of WT-CagA on Runx3 expression most likely occur at the transcriptional level.

### PR-CagA CANNOT AFFECT THE EXPRESSION OF Runx3 IN GES-1 CELL

Next, we want to investigate whether the inhibition is dependent on the CagA tyrosine phosphorylation. We used the PR-CagA plasmid to transfect GES-1 cell. The PR-cagA plasmid encodes a CagA protein with a mutation in the EPIYA motif, required for CagA tyrosine phosphorylation. Thus the PR-CagA mutant protein cannot be phosphorylated nor can it transmit the signal pathway [Zhu et al., 2004, 2005; Yokoyama et al., 2005]. After 48 h transfection of PR-CagA plasmid into GES-1 cell, we used Western blot to detect the tyrosine phosphorylation of CagA with the PY99 antibody. The result illustrated in Figure 1A showed that the CagA cannot be phosphorylated in PR-CagA transfected cell, which is consistent with the result of Zhu's [Zhu et al., 2004]. Then QRT-PCR and

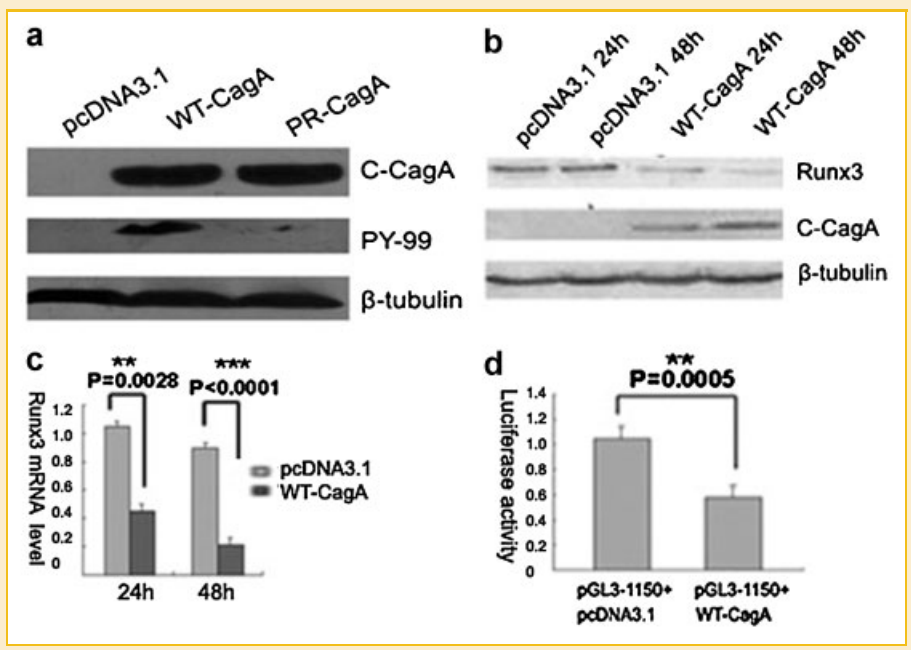


Fig. 1. Analysis of WT-CagA transfection and its effects on Runx3 expression. a: GES-1 gastric epithelial cells were transfected with blank vector pcDNA3.1, WT-cagA plasmid and PR-CagA plasmid for 48 h, respectively. Total cellular protein was extracted for Western blot analysis for the protein expression of C-CagA and PY99. b: GES-1 cells were transfected with blank vector pcDNA3.1 or WT-cagA plasmid for 24 and 48 h, respectively. Total cellular protein was extracted for Western blot analysis for the protein expression of CagA and Runx3. c: GES-1 cells were transfected with pcDNA3.1 or WT-cagA plasmid for 24 or 48 h. Total cellular RNA was extracted for QRT-PCR analysis for the mRNA level of Runx3. d: Effect of WT-CagA over-expression on Runx3 promoter activity. GES-1 cells were transfected with pGL3-1150 and pcDNA3.1 or WT-CagA. Forty-eight hours after transfection, the cells were harvested for a dual-luciferase assay. pRL-TK vector was used to correct for the transfection efficiency. Results are expressed as the ratio of firefly luciferase activity (M1) in the pGL3 plasmid to Renilla luciferase activity (M2) in the pRL-TK plasmid. Data are presented as the mean of four individual values ±SD.

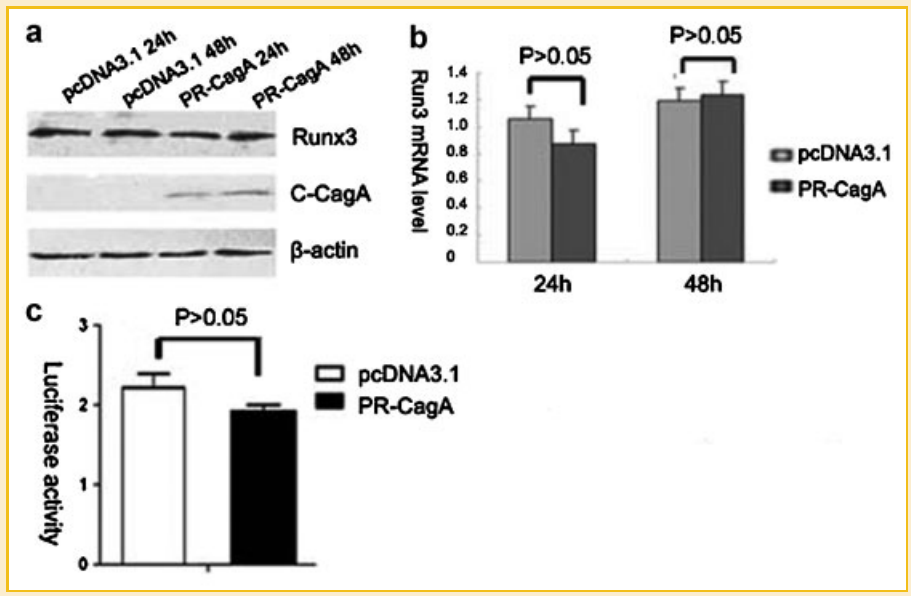


Fig. 2. Analysis of PR-CagA transfection and its effects on Runx3 expression. a: GES-1 gastric epithelial cells were transfected with pcDNA3.1 or PR-cagA plasmid for 24 and 48 h, respectively. Total cellular protein was extracted for Western blot analysis for the protein expression of CagA and Runx3. b: GES-1 cells were transfected with pcDNA3.1 or PR-cagA plasmid for 24 or 48 h. Total cellular RNA was extracted for QRT-PCR analysis for the mRNA level of Runx3. c: Effect of PR-CagA over-expression on Runx3 promoter activity. GES-1 cells were transfected with pGL3-1150 and pcDNA3.1 or PR-CagA. 48 h after transfection, the cells were harvested for a dual-luciferase assay. pRL-TK vector was used to correct for the transfection efficiency. Results are expressed as the ratio of firefly luciferase activity (M1) in the pGL3 plasmid to Renilla luciferase activity (M2) in the pRL-TK plasmid. Data are presented as the mean of four individual values ±SD.

Western blot were used to determine the regulatory effect of PR-CagA on Runx3 expression after 24 and 48 h transfection. The results illustrated in Figure 2 showed that PR-CagA has a very high expression level after transfection (Fig. 2A) and PR-CagA cannot affect the expression of Runx3 both at RNA and protein levels (Fig. 2A,B). Then the luciferase reporter assay was used to determine whether PR-CagA can affect the promoter activity of Runx3. Figure 2C showed that the activity of the *Runx3* promoter cannot be inhibited by PR-CagA significantly, which is consistent with the results of QRT-PCR and Western blot. Therefore, these results suggested that the regulatory effect of CagA on Runx3 is dependent on the phosphorylation of CagA protein in its Tyr site. Also we can infer that the regulatory effect of WT-CagA on Runx3 may depend on the interaction between SHP-2 and the phosphorylated CagA protein since the Src-homology 2 (SH2) domains of the protein tyrosine phosphatase SHP-2 can only interact with the phosphorylated CagA [Higashi et al., 2002].

### THE EFFECT OF SIGNAL INHIBITOR ON Runx3 EXPRESSION IN GES-1 CELLS TRANSFECTED WITH WT-CagA PLASMID

We next wanted to determine the pathway(s) through which CagA affected Runx3. For this purpose, we used the PP1 to block Src-family tyrosine kinase activity [Hanke et al., 1996], the MAP kinase inhibitor PD98059 to block MEK1 kinase activity, thereby inhibiting ERK1/2 phosphorylation [Alessi et al., 1995], SB203580 to block p38 kinase activity [Young et al., 1997], BAY11-7082 to inhibit the NF-kB pathway [Pierce et al., 1997]. Each signal inhibitor was incubated with gastric cells for 30 min prior to plasmid transfection. After 48 h transfection, QRT-PCR, Western blot, and luciferase activity assay were used to determine the Runx3 level. Figure 3 showed that Runx3 down-regulation by WT-CagA was attenuated by PP1 (Src kinase inhibitor), SB203580 (P38 kinase inhibitor), and PD98059 (MEK1 inhibitor) at transcription level and Runx3 down-regulation was maintained using BAY11-7082 (NF-kB inhibitor). In total, the results indicated that Src kinase and MEK/ERK pathways participated in the down-regulation of Runx3 by CagA. In GES-1 cells, CagA protein was tyrosine phosphorylated by Src kinase, thereafter promoting the activated MEK/ERK pathway to down-regulate Runx3 expression [Backert and Meyer, 2006].

In order to investigate whether WT-CagA can induce the ubiquitination and degradation of RUNX3 in GES-1 cell, we used MG-132 (10 μM) to treat the cells for 6 h [Tsang et al., 2010] and Western blot was used to determine the Runx3 expression. The result illustrated in Figure 4 showed that MG-132 cannot attenuate the down-regulation of Runx3 induced by WT-CagA. But when we transfected AGS cells with Runx3/pcDNA3.1 and WT-CagA plasmid, after 36 h, we used MG-132 to treat the transfected cells, we found that the exogenous Runx3 level was reduced, which is consistent with Tsang's result (Data not shown).

## DISCUSSION

*Helicobacter pylori* has been defined as a class I carcinogenic factor and its persistent colonization in the stomach increases the risk of gastric cancer [Peek and Blaser, 2002; Houghton and Wang, 2005]. CagA is one of the most important virulence factors secreted by *H. pylori*. CagA can interact with various host cellular proteins to trigger distinct signaling pathways in a tyrosine phosphorylation-

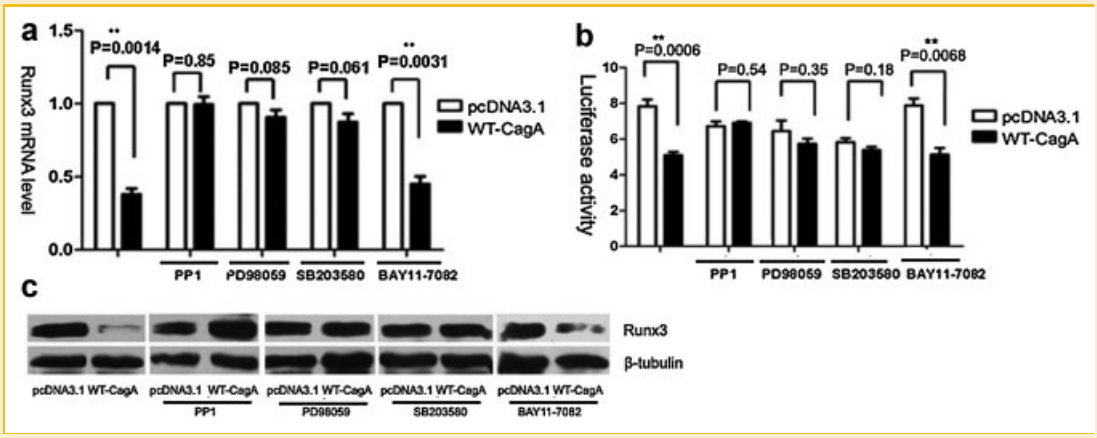


Fig. 3. Effects of signal inhibitors on CagA-induced Runx3 down-regulation in GES-1 cells. a: GES-1 cells were incubated with Src-family tyrosine kinase inhibitor PP1 (20 μM), MEK1 inhibitor PD98059 (50 μM), p38 MAP kinase inhibitor SB203580 (10 μM) or NF-kB pathway inhibitor BAY11-7082 (5 μM) for 30 min, respectively. Then the cells were transfected with pcDNA3.1 or WT-cagA plasmid for 48 h. Total cellular RNA was extracted for QRT-PCR analysis for the mRNA level of Runx3. b: GES-1 cells were treated with different signal inhibitor mentioned in (a) for 30 min. Then the cells were transfected with pGL3-1150 and pcDNA3.1 or WT-CagA plasmid for 48 h. After transfection, the cells were harvested for a dual-luciferase assay. pRL-TK vector was used to correct for the transfection efficiency. Results are expressed as the ratio of firefly luciferase activity (M1) in the pGL3 plasmid to Renilla luciferase activity (M2) in the pRL-TK plasmid. Data are presented as the mean of four individual values ±SD. c: GES-1 cells were treated with different signal inhibitor mentioned in (a) for 30 min. Then the cells were transfected with blank vector pcDNA3.1 or WT-cagA plasmid for 48h. Total cellular protein was extracted for Western blot analysis for the expression of Runx3.

dependent and -independent manner [Hatakeyama, 2004; Peek, 2005].

The transcription factor Runx3 was suggested to be a tumor suppressor in gastric cancer and closely associated with tumorigenesis and progression of gastric cancer [Li et al., 2002; Sakakura et al., 2005]. In most of the normal gastric mucosal epithelial cells, the expression level of Runx3 is high, but in nearly 90% of advanced stage gastric carcinomas and most of the gastric cancer cell lines, such as AGS, MKN28, MKN45, KATOIII, the expression level of Runx3 is very low or completely inactivated. Thus, it is far from certain that the relationship between *H. pylori* CagA and Runx3 are accurately reflected in these malignant cell lines. In this study, we used an immortalized fetal gastric mucosa cell line transformed with SV40. The cell line was established by Ke et al. [1994]. It has a phenotype similar to that of normal stomach mucosal cells and Runx3 has a higher expression level in this cell line than in other gastric cancer cell lines [Guo et al., 2005]. Therefore, it was hoped that our results could display the interaction of *H. pylori* CagA and Runx3 more accurately.

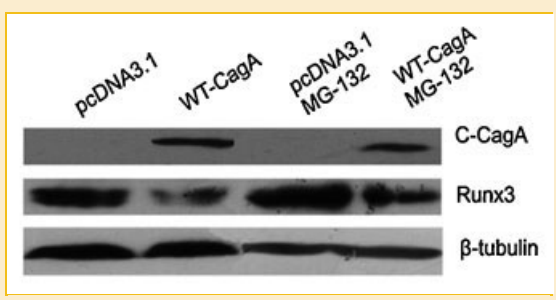


Fig. 4. Effect of MG-132 on WT-CagA induced down-regulation of Runx3. GES-1 cells were transfected with plasmid WT-cagA or pcDNA3.1 control vector. At 36 h after transfection, cells were treated with or not with MG-132 (10 μM) for 6 h. Total cellular protein was extracted for Western blot analysis for the expression of Runx3.

Tsang et al. [2010] showed that *H. pylori* infection inactivates RUNX3 in a CagA-dependent manner. CagA can directly associate with RUNX3 and induce the ubiquitination and degradation of RUNX3 in cytoplasm in AGS cell. In order to investigate whether WT-CagA can also induce the ubiquitination and degradation of endogenous RUNX3 in GES-1 cell, we transfected GES-1 cells with plasmid WT-cagA or pcDNA3.1 control vector. Then the cells were treated with or not with MG-132 (10 μM). We used Western blot to determine the Runx3 expression. The result illustrated in Figure 4 showed that WT-CagA can also reduce the expression level of Runx3 in MG-132 treated cells and MG-132 did not attenuate the down-regulation of Runx3 induced by WT-CagA. We think that the degradation of RUNX3 in cytoplasm was cell specific. Since GES-1 cells express endogenous RUNX3, maybe it is different from AGS that has no endogenous RUNX3 expression.

As a transcription factor, most of the RUNX3 protein exercises its function in the cell nucleus. Whether CagA can affect intra-nuclear Runx3 expression through some signal pathway(s) is still not clear. In this study, we found that in GES-1 cell, the wild type CagA (WT-CagA) can down-regulate Runx3 expression significantly, but the phosphorylation-resistant plasmid derivative (PR-cagA) has no effect on the expression of Runx3. According to the results, we concluded that CagA can down-regulate Runx3 expression in a tyrosine phosphorylation-dependent manner. Since the C terminus of CagA can be tyrosine phosphorylated by Src kinase, the phosphorylated CagA involves in MEK/ERK pathway, p38 MAP kinase pathway and NF-kB pathway [Backert and Meyer, 2006]. It was previously shown that signal inhibitors could be used to elucidate which pathway(s) was involved in CagA-induced IL-8 production [Kim et al., 2006] and CagA-induced CIP2A expression

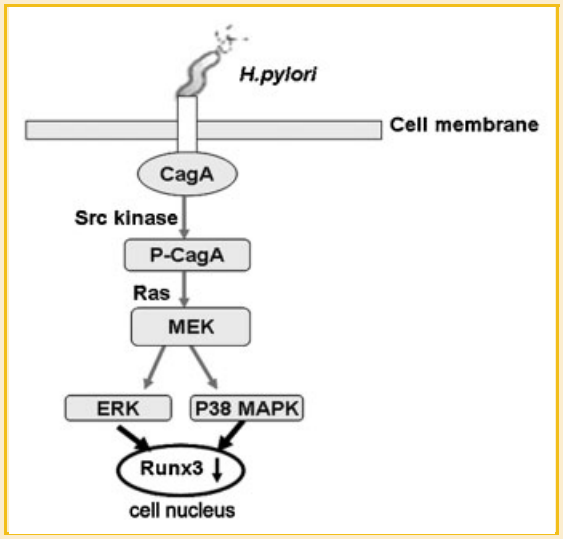


Fig. 5. Schematic model of the down-regulation effect of *H. pylori* CagA on Runx3. Bacterial oncoprotein CagA was translocated into gastric epithelial cells through the TFSS, and then activated Src kinase and was phosphorylated by Src kinase. After that, P-CagA activated the MEK/ERK and P38 MAPK pathway and down-regulated gastric tumor suppressor Runx3 expression.

[Zhao et al., 2010]. In order to elucidate which signal pathway(s) involve in the regulation, we used four signal inhibitors to block corresponding signal transduction. We found that Src kinase, MEK/ERK or P38 MAPK pathways inhibitors can block the regulatory effect of CagA on Runx3 while NF-kB pathway inhibitor has no such effect. So we concluded that Src kinase, MEK/ERK and MEK/P38 MAPK pathways participated in the regulation of Runx3 by CagA. Based on our findings, we figured out the regulation mechanisms of *H. pylori* CagA on the expression of Runx3 (Fig. 5). Upon delivery into gastric epithelial cells through the TFSS, *H. pylori* CagA was tyrosine-phosphorylated by Src kinase and the P-CagA activated the MEK/ERK pathway or MEK/P38 MAP kinase pathway, leading to the down-regulation of gastric tumor suppressor RUNX3 expression.

In summary, gastric tumor suppressor Runx3 can be down-regulated by *H. pylori* CagA via the Src/MEK/ERK and p38 MAPK pathways. The down-regulation of Runx3 may accelerate the progression of gastric diseases from chronic gastritis to gastric cancer. This study contributed to understand the mechanism by which gastric tumors are caused by *H. pylori* infection in humans.

## ACKNOWLEDGMENTS

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