Original Article

Repressed TGF-β signaling through CagA-Smad3 interaction as pathogenic mechanisms of *Helicobacter pylori*-associated gastritis

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Helicobacter pylori* (*H. pylori*) infection causes chronic gastric inflammation, peptic ulceration, and gastric carcinogenesis, in which *H. pylori* cytotoxin-associated gene A (CagA) plays major pathogenic action. Since transforming growth factor-β (TGF-β) and its signaling also are principally implicated in either modulating gastric mucosal inflammatory responses or causing carcinogenesis and are attenuated after *H. pylori* infection, we hypothesized that dysregulated Smad signaling and repressed TGF-β might be core pathogenic mechanism for *H. pylori*-associated gastritis or carcinogenesis. Until now, no precise underlying mechanism how deranged TGF-β signaling developed after *H. pylori* infection relevant to various clinical manifestations remains unclear. In this study, we examined the molecular mechanism about the inhibition of TGF-β signaling by *H. pylori* CagA protein. *H. pylori* CagA significantly suppressed TGF-β/Smad transcriptional responses through critical inhibition of Smad3, though CagA interacted constitutively with Smad2, Smad3, and Smad4. CagA inhibited TGF-β-induced suppression of proinflammatory chemokines, such as IL-8, CXCL1 and CXCL3, as well as TGF-β-induced transcription of target genes. In conclusion, repressed TGF-β signaling associated with CagA-positive *H. pylori* infection could be an important determinant for the outcome of *H. pylori* infection. Therefore, TGF-β signaling is one of the important determinants to avoid from *H. pylori* CagA pathogenicity.

***Key Words*:** ***Helicobacter pylori*, Cytotoxin-associated gene A, TGF-β, Smad, inflammation**

Helicobacter pylori (*H. pylori*) infection is associated with gastritis, gastric atrophy, and mucosa-associated lymphoid tissue (MALT) lymphoma in the stomach and *H. pylori*-associated chronic inflammation is basis for gastric cancer,[1] by which defined as class I carcinogen by IARC (Lyon, France). *H. pylori*, a Gram negative, spiral-shaped microaerophilic pathogen, is extremely variable and its strain differs markedly in many aspects such as adherence to the gastric mucosa and ability to provoke inflammation.[2,3] *H. pylori* colonization occurs in childhood and persists throughout life, causing disease mainly in adults because it can adapt to human colonization and produces disease-inducing factors including urease, cytotoxin, catalase, and lipopolysaccharide (LPS).[4] A significant increase in the levels of proinflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were detected when infected with *H. pylori* or stimulated with *H. pylori* LPS.[5] *H. pylori* enhanced the risk for gastric cancer by increasing STAT3 signaling in epithelial cells,[6,7] and disrupt IL-4-mediated STAT6 signaling in epithelial cells and inhibit a Th2 immune response, prerequisite for eliminating the pathogen.[8]

H. pylori strains can be divided into two major subpopulations based on their ability to produce a 120–145 kDa immunodominant protein called cytotoxin-associated gene A (CagA) antigen.[9] Compared with CagA negative *H. pylori* strains, CagA positive strains more increases the risk of developing gastric inflammation, atrophic gastritis, peptic-ulcer disease and gastric carcinoma. Upon attachment of CagA (+) *H. pylori* to the gastric epithelial cell, the CagA protein is delivered directly into the cell via the cag PAI-encoded type IV secretion system.[10] Then, the translocated CagA localizes to the inner surface of the plasma membrane, where it undergoes tyrosine phosphorylation at EPIYA (Glu-Pro-Ile-Tyr-Ala) motifs by several members of the *Src* family kinases.[11,12] CagA is capable of controlling transcription factors via both phosphorylation-dependent and -independent mechanisms, generating deregulated signals for cell growth, cell-cell contact and cell movement.[13,14] Therefore CagA may cause proliferation, apoptosis or differentiation, depending on the cellular setting and induces the hummingbird phenotype which is characterized by elongated cell-shape with dramatic cytoskeletal rearrangements.[9]

Transforming growth factor β (TGF-β) is one of the most widely distributed cytokines that acts on virtually all cell types and mediates highly pleiotropic functions.[15] TGF-β is able to regulate proliferation, differentiation, motility and apoptosis and plays an important role in the control of immune homeostasis and prevention of mucosal inflammation.[16,17] Therefore, TGF-β consistently has been linked to several human pathogenic processes such as fibrosis, inflammation and carcinogenesis. TGF-β signals are transduced by transmembrane serine-threonine kinase receptors and intracellular effectors Smads.[18,19] Upon the binding of TGF-β to its receptors, Smad2 and/or Smad3 are phosphorylated at their C-termini by the type I receptor. The phosphorylated Smad2/3 are engaged in a complex with Smad4 and then translocated into the nucleus.

Impairment mutations in components of the TGF-β signaling network, in particular in the TGF-β type II receptor, commonly occur in gastrointestinal (GI) tumors.[20,21] Similarity, loss of the bone morphogenic protein (BMP) type IA receptor or mono-allelic germline mutations in the Smad4 gene predispose to the hamartomatous, GI familial juvenile polyposis syndrome in human,[22,23] whereas mono-allelic null mutations in either *Tgfb1* (which encodes TGF-β) or Smad4, or hypomorphic Smad4 mutations, cause late-onset gastric polyposis in mice.[24,25] In addition, TGF-β is capable of modulating inflammatory responses by inhibiting the proliferation of B- and T-lymphocytes and suppressing macrophages and natural killer cell activity.[26,27] TGF-β1 knockout mice develop a severe multiple organ inflammatory

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disease, mostly involving the stomach and the intestine.[28] Interestingly, the gastric findings noted in TGF-β1-deficient mice show similar pathologies to those observed in *H. pylori* infection such as hyperplasic gastritis and gastric dysplasia. Indeed, conditional loss of TGF-β1 activity selectively in the gastric mucosa of mice is associated with exaggerated and severe inflammation and prominent proliferation after *H. pylori* infection.[29] Though loss of TGF-β exhibits a similar pathology to that seen in a subset of individuals infected with *H. pylori*, including propagated gastric inflammation, oxidative stress, and autoimmune features,[30] the link between TGF-β signaling and *H. pylori* has not been clear. In this study, we examined the molecular mechanism of *H. pylori* CagA protein to suppress TGF-β signaling.

Materials and Methods

Cell culture. AGS (human gastric adenocarcinoma) cells were grown in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gibco) and 1% mixture of penicillin and streptomycin (Gibco). 293T (human embryonic kidney) cells, RGM-1 (rat gastric mucosal) cells, MKN28 and MKN45 (human gastric adenocarcinoma) cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum and 1% mixture of penicillin and streptomycin (Gibco). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Bacterial strain. *H. pylori* strain (CagA+ strain ATCC 43504) was obtained from ATCC (Rockville, MD). Concentrations of *H. pylori* were estimated, using OD 600 of 1 as 1×10^8 bacteria/ml.[5,30]

Plasmid constructs. Control plasmid pSP65SRα, hemagglutin (HA)-tagged CagA expression plasmid (pSP65SRα-WT-CagA-HA), and HA-tagged phosphorylation-resistant (PR) CagA expression plasmid (pSP65SRα-PR-CagA-HA) were a generous gift from Dr. Hatakeyama Masanori.[31] Flag-tagged Smad2, Smad3, Smad4, and Flag-tagged Smad3 deletion constructs were described previously.[32,33]

Transfection and reporter assays. AGS cells were transiently transfected with SBE4-Luc, 3TP-Lux, ARE-Luc together with forkhead activin signal transducer (FAST)-1, BRE-Luc and the internal control pCMV-β-gal in 24-well plate using PEI reagent. After 24 h transfection, cells were treated with 5 ng/ml TGF-β1 for 16 h. In case of *H. pylori* infection experiment, cells were infected with the indicated amount of *H. pylori* in 4 h before TGF-β1 treatment. Luciferase activity was quantified by using Luciferase Assay Substrate Kit (Promega Corp., Madison, WI). Values were normalized with the β-galactosidase activity. All experiments were performed in triplicate and repeated at least three times.

Immunoblotting and immunoprecipitation. AGS cells or 293T cells were used for the detection of protein-protein interaction *in vivo*. Cells were transiently transfected with the indicated plasmids. After 24 h transfection, AGS cells were treated with 5 ng/ml TGF-β1 for 1 h. Cells were lysed in a buffer containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, and protease inhibitor mixture (Complete, Roche Diagnostics, Basel, Switzerland). Extracts were separated by SDS-PAGE followed by electro-transfer to polyvinylidene difloride (PVDF) membranes and probed with polyclonal or monoclonal antisera, followed by horseradish peroxidase-conjugated anti-rabbit, anti-mouse IgG and visualized by chemiluminescence, according to the manufacturer's instructions (Pierce, Rochford, IL). For immunoprecipitation the cell lysates were incubated with the appropriated antibody for 1 h, followed by incubation with Protein A Excellose-binding bead (Bioprogen, Daejeon, Korea) for 1 h at 4°C. Beads were washed four times with the buffer used for cell solubilization. Immune complexes were then eluted by boiling for 5 min in 2X Tris-Glycine SDS Sample Buffer (Invitrogen Corp., Carlsbad, CA), and then extracts were analyzed by immunoblotting as described above.

Reverse Transcription-PCR. Total RNA was isolated from AGS cells using RNA isoplus reagent (Takara Bio Inc., Shiga, Japan), and 2 μg of each total RNA was converted to cDNA using the M-MLV Reverse Transcriptase (Promega) system for RT-PCR using Oligo-dT primer. Primers such as CagA, Smad7, PAI-1 (plasminogen activator inhibitor-1), c-Myc, fibronectin, Id (DNA-binding protein inhibitor) 1, CXCL (CXC chemokine ligand) 1, CXCL2, CXCL3, IL-8 (interleukin-8 or CXCL8) and GAPDH were used for PCR.

Confocal microscopy. AGS cells were seeded into 4-well chamber slides. Then cells were transfected with Flag-Smad3 and HA-CagA for 24 h. Before harvesting, cells were treated TGF-β1 for 1 h. Harvested cells were washed in cold phosphate-buffered saline with 1% fetal bovine serum, fixed in 4% PFA, and then stained with anti-Flag antibody (F1804) (Sigma Aldrich, St. Louis, MO) and anti-HA antibody (sc-805) (Santa Cruz Biotechnology, Santa Cruz, CA) followed by Alexa Fluor goat anti-mouse (Invitrogen Corp.) and Alexa Fluor goat anti-rabbit (Invitrogen Corp.) in a solution containing PBS, 1% fetal bovine serum, 0.2% saponin, and 3% cold fish gelatin (Sigma Aldrich). After the final wash, the cells were resuspended in a minimal volume of mounting solution, placed on a slide, covered with a coverslip, and the edges sealed with nail polish. Cells were observed on a LSM-710 confocal microscope (Zeiss, Oberkochen, Germany).

Statistical analysis. Results in bar graphs are presented as means ± SD and are representative of three independent experiments. Statistical analysis was performed using the Student's *t* test, and *p* values of less than 0.05 were considered statistically significant.

Results

***H. pylori* infection specifically inhibited the TGF-β1-induced transcriptional activation.** To test whether *H. pylori* infection affects TGF-β signaling, AGS cells were transfected with SBE4-Luc and 3TP-Lux reporter construct. After 24 h transfection, cells were infected with *H. pylori* in different multiplicity of infection (MOI) such as 5, 50, and 500. Following by TGF-β1 was treated for 12 h before harvesting, we have observed the activity of SBE4-luc and 3TP-lux, respectively. As seen in Fig. 1A and B, a significant decrement in relative luciferase unit of SBE4-Luc and 3TP-lux was noted along with increment in *H. pylori* MOI ($p<0.05$). To further test whether *H. pylori* CagA is responsible for repressing the TGF-β-induced transcriptional activation, AGS cells were transiently co-transfected with HA-CagA, ARE-Luc reporter construct together with FAST-1 and BRE-Luc reporter assays were done, respectively. After 24 h transfection, cells were treated with either TGF-β1 (Fig. 2C) or BMP-2 (Fig. 2D) in 16 h, respectively. As seen in Fig. 2C and D, similar results were drawn as with *H. pylori* infection or CagA transfection. However, significant inhibition of TGF-β1-induced transcriptional activation was noted with CagA, whereas CagA didn't have any effect on BRE-Luc reporter assay which is specific for BMP signaling (Fig. 2D).

Both wild type and mutant CagA could repress TGF-β1-induced transcriptional activation. To test whether *H. pylori* CagA can repress the TGF-β-induced transcription activation, AGS cells were transiently co-transfected with HA-CagA and SBE-Luc or 3TP-Lux and the reporter activities were measured. As anticipated from Fig. 1, *H. pylori* CagA significantly decreased SBE-Luc and 3TP-Lux promoter activities ($p<0.05$). Additionally, we also checked whether transcriptional inactivation of TGF-β after *H. pylori* infection can be seen in other gastric epithelial cell lines, RGM-1, MKN28, and MKN45 cells and found same experimental results (data not shown). Then, the next hypothesis whether only wild type *H. pylori* CagA was responsible for these

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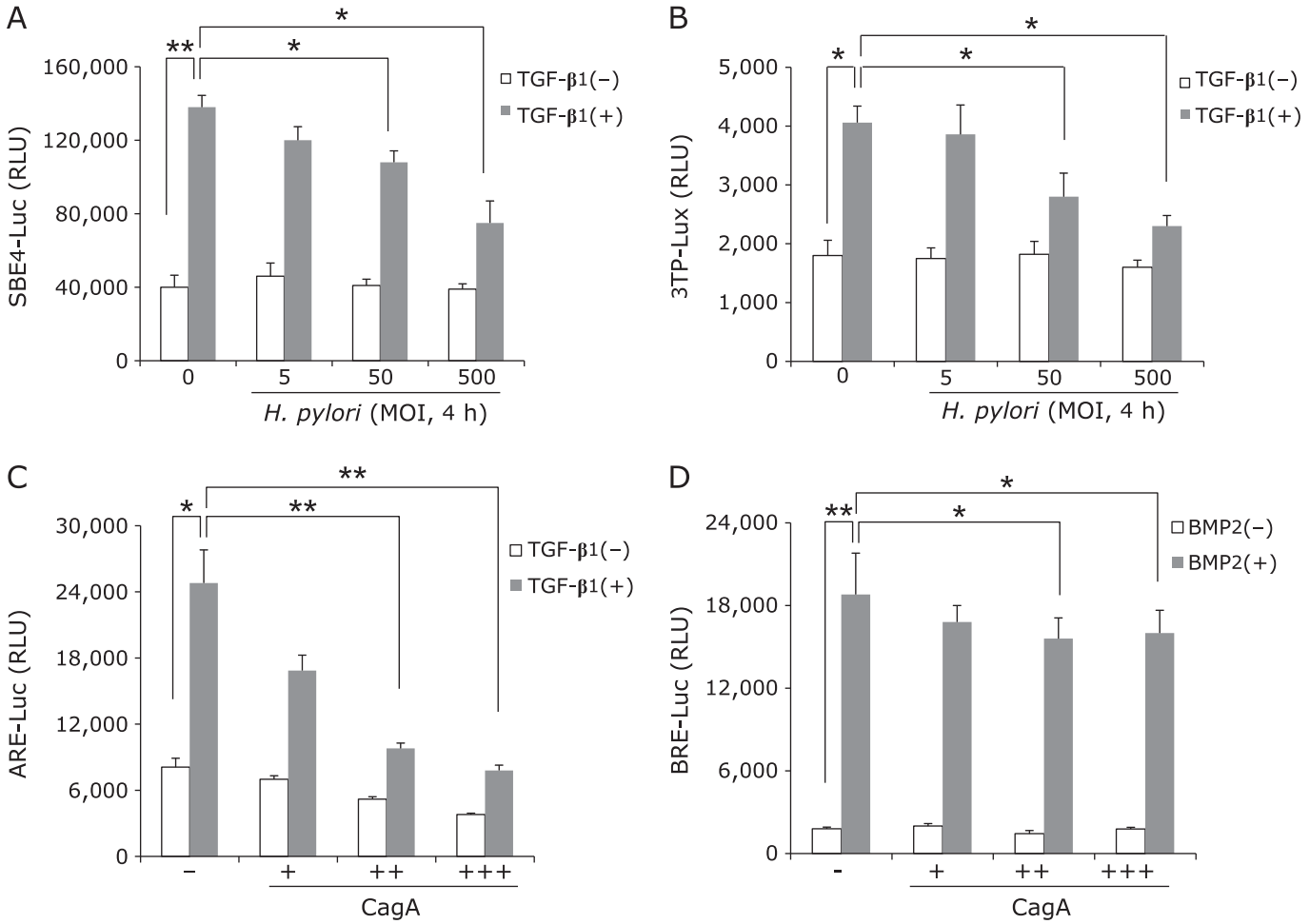

Fig. 1. Infection of *H. pylori* inhibits TGF-β-induced transcriptional activation. AGS cells transfected with SBE4-Luc (A) or 3TP-Lux (B) were infected with *H. pylori* [ATCC 43504, CagA (+) strain] for 4 h at different MOIs. Luciferase activity was measured after 16 h of TGF-β1 stimulation. CagA was co-transfected into AGS cells with ARE-Luc together with FAST-1 (C) or BRE-Luc (D). Luciferase activity was measured after 16 h of TGF-β1 or BMP-2 stimulation. Data shown are the mean ± SD of three separate experiments. *p<0.05, **p<0.01.

transcriptional inactivation of TGF-β was put, for which we repeated assay after transfection with a mutant CagA (ABccc) construct in which tyrosine residues in three EPIYA-C motifs were replaced by alanine. As seen in Fig. 2C and D, mutant CagA (ABccc) also led to the transcriptional inactivation of TGF-β.

***H. pylori* CagA repressed the Smad transcriptional activity and interacts with Smads.** Then, we determined whether CagA can directly suppress Smad transcriptional activity, for which we used a heterologous reporter assay. The GAL4 DNA-binding domain was fused to various Smad proteins. GAL4-Smad2, GAL4-Smad3, or GAL4-Smad4 expression constructs were cotransfected with a luciferase reporter construct (G5E1b-Lux), which contained five GAL4-binding sites upstream of the AdE1b TATA box. As shown in Fig. 3A, TGF-β1 treatment did not induce transcription by the minimal GAL4-DNA binding domain, and CagA did not have any effect on its transcription. However, CagA strongly suppressed TGF-β1-induced transcriptional activity of GAL4-Smad3 fusion proteins (p<0.05, Fig. 3A), demonstrating that CagA can directly suppress Smad-mediated transcriptional activation. Therefore, in order to verify these findings, 293T cells were co-transfected with HA-CagA and Flag-Smad2, Flag-Smad3, or Flag-Smad4, respectively. Cell extracts were immunoprecipitated with anti-HA antibody and immunoblotting with anti-Flag antibody was done (Fig. 3B). As results, CagA interacted the most strongly with Smad3, lesser with Smad4 and weakly with Smad2. To ensure the interaction between HA-CagA and Flag-Smad3, we did the particular immunoprecipitation experiment using only two constructs including HA-CagA and Flag-Smad3. As shown in Fig. 3C, CagA clearly interacted with Smad3 as demonstrated by immunoprecipitation with anti-HA antibody and detected with anti-Flag antibody and *vice versa*. With all of these immunoprecipitated binding assay, we reached to the conclusion that *H. pylori* infection led to the clear interaction of CagA-Smad3 and subsequent inhibition of TGF-β signaling through CagA-Smad3 binding.

***H. pylori* CagA interacted with MH2 domain of Smad3.** To further investigate which domain of Smad3 directly interacts with CagA *in vitro*, we performed immunoprecipitation assay using various Flag-Smad3 deletion constructs, namely MH1, MH1 + L, L + MH2, and MH2 (Fig. 4A) and HA-CagA construct. As seen in Fig. 4B, the *N*-terminal mutants in which contains L + MH2 or MH2 clearly interacted with CagA, whereas the C-terminal which have MH1 or MH1 + L were unable to bind to CagA. The linker region is not likely to interact with CagA because MH1 + L did not interact with CagA. These results showed that the MH2 domain of Smad3 contained an important CagA interaction domain for TGF-β signaling.

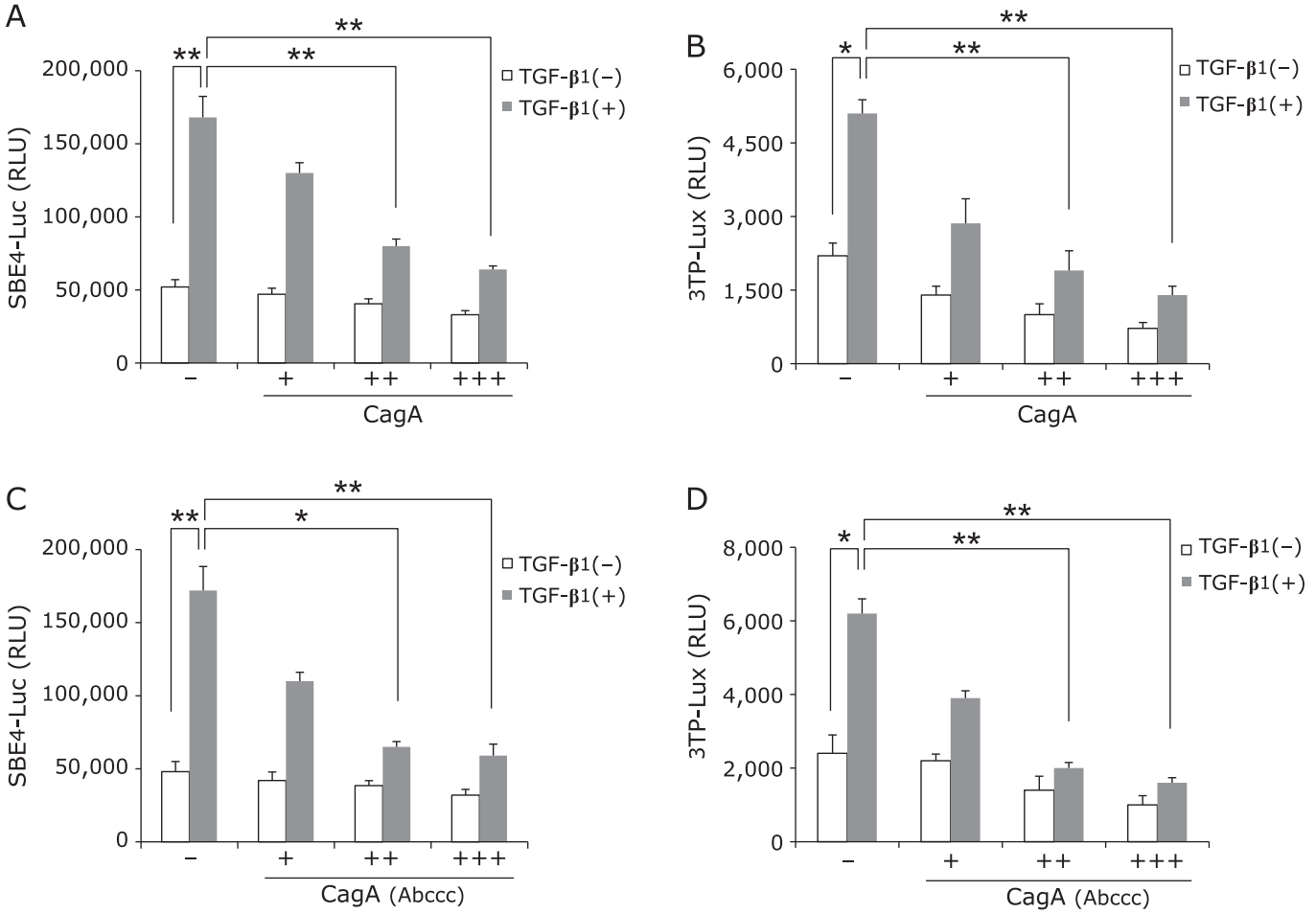

Fig. 2. CagA inhibits TGF-β-induced transcriptional activation. CagA was co-transfected into AGS cells with SBE4-Luc (A), 3TP-Lux (B). Mutant CagA (ABccc) was co-transfected into AGS cells with SBE4-Luc (C), 3TP-Lux (D). Luciferase activity was measured after 16 h of TGF-β1 stimulation. Data shown are the mean ± SD of three separate experiments. *p<0.05, **p<0.01.

***H. pylori* CagA inhibited TGF-β-induced nuclear translocation of Smad3 and suppressed the endogenous complex formation of Smad3-Smad4.** The transcriptional activities of both Smad2 and Smad3 are dependent on their phosphorylation by activated TGF-β type I receptor. Therefore, we examined whether CagA regulates TGF-β-stimulated Smad2 and Smad3 phosphorylation. CagA expression had little effect on TGF-β-stimulated phosphorylation of endogenous Smad2 and Smad3 in AGS cells expressing CagA compared to the control cells, suggesting that effects of CagA are positioned downstream of Smad2 and Smad3 phosphorylation (Fig. 5A). To check the cellular localization of CagA and Smad3, confocal microscopy was performed in AGS cell with the transiently transfection HA-CagA and Flag-Smad3. As shown in Fig. 5B, HA-CagA and Flag-Smad3 were found in cytoplasmic in non TGF-β1 treatment. Upon TGF-β1 stimulation, only Smad3 was translocated into the nucleus, but when we co-transfected with HA-CagA and Flag-Smad3 following by treatment TGF-β1, mostly either HA-CagA or Flag-Smad3 were still localized in cytoplasm. As CagA interacts with Smad3 and Smad4, we examined whether CagA might inhibit the endogenous complex formation of Smad3 and Smad4. AGS cells were transfected control vector pSp65SRα and HA-CagA. After 24 h transfection, cells were incubated in the presence or absence of TGF-β1 for 1 h. Then total cell extracts were immunoprecipitated with anti-Smad3 antibody. The Smad3-bound Smad4 was detected by Western blot analysis using anti-Smad4 antibody. The level of Smad3-bound Smad4 was significantly decreased in AGS transfected with HA-CagA comparing with AGS transfected with control vector cells (Fig. 5C).

***H. pylori* CagA interrupted the anti-inflammatory function of TGF-β.** We next examined the effect of CagA on the expression of some typical genes induced by TGF-β1. First, AGS cells were respectively transfected control vector and HA-CagA in the present or absent of TGF-β1 for 8 h. Then total RNA was isolated and performed RT-PCR. As seen in Fig. 6A, the expression level of PAI-1, Id1, fibronectin was significantly increased with TGF-β1 treatment, whereas no significant changes were noted in the presence of CagA alone. However, CagA presence did not increase those gene expressions relevant to 8 h TGF-β1 treatment (Fig. 6A), compatible with abrogated TGF-β1-relevent gene expression in the presence of *H. pylori* CagA gene. It is well known that TGF-β has an important role in anti-inflammation, whereas *H. pylori* CagA is responsible for inducing many inflammation cytokine. Therefore, we test whether CagA can abolish the anti-inflammatory function of TGF-β. As seen in Fig. 6B, *H. pylori* CagA increased the expression of IL-8, CXCL1, and CXCL3. However, CagA abrogated the TGF-β1-induced suppression of IL-8, CXCL1, CXCL2, and CXCL3, signifying that when TGF-β signaling was significantly suppressed by *H. pylori* infection, leading to the conclusion that *H. pylori* CagA-Smads

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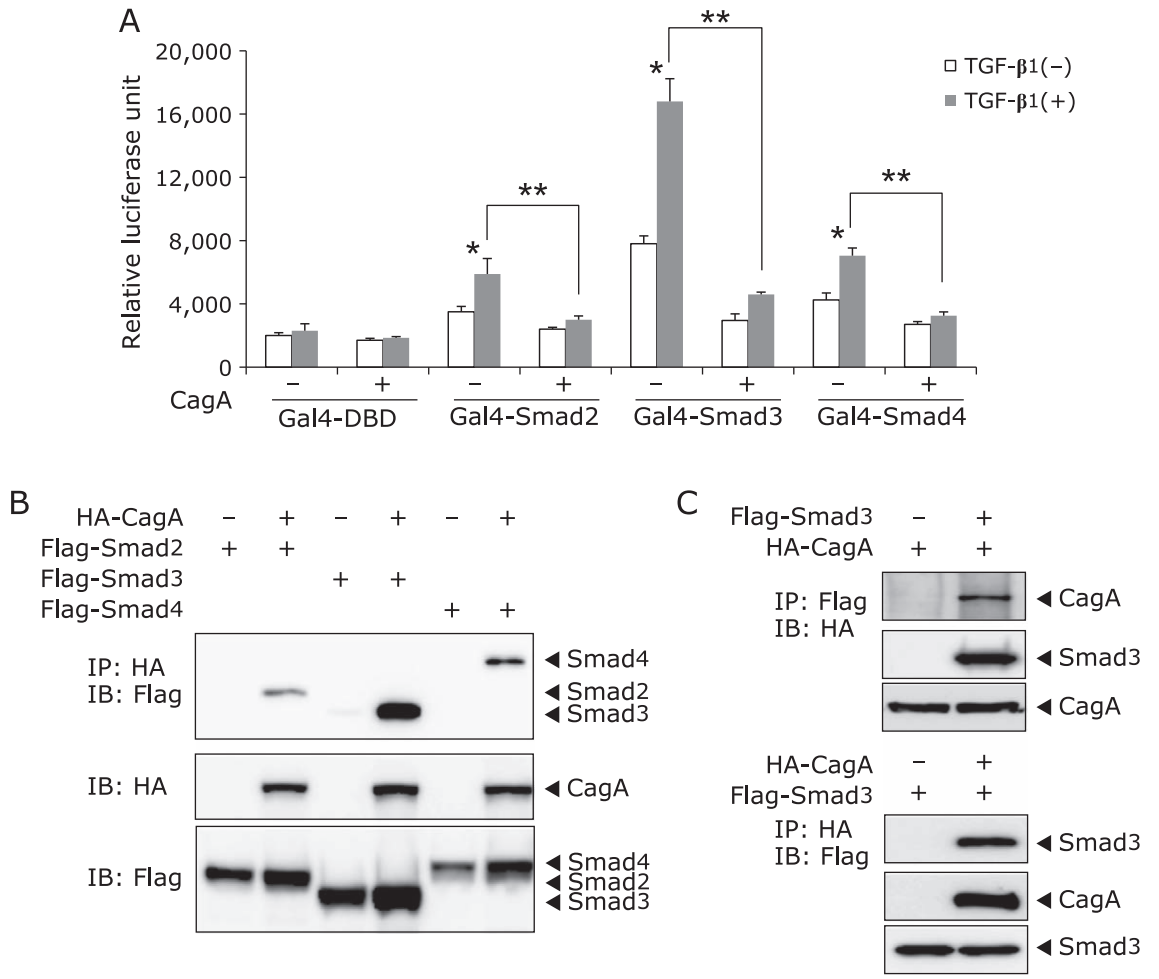

Fig. 3. CagA inhibits Smad transcriptional activity and interacts with Smad proteins. (A) AGS cells were cotransfected with GAL4 fusion constructs together with G5E1b-Lux in the presence or absence of CagA. Cells were treated with or without TGF-β1 for 16 h. Cell lysates were analyzed for luciferase activity. Data shown are the mean ± SD of three separate experiments. *p<0.05 compared to Gal4DBD. **p<0.05 compared to Gal4-Smad. (B) HEK293T cells were transfected with HA-tagged CagA and Flag-tagged Smad2, Smad3 or Smad4. Protein lysates were immunoprecipitated with anti-HA and immunoblotted with anti-Flag antibody. (C) HEK293T cells were transfected with Flag-tagged Smad3 and HA-tagged CagA. CagA was detected in Smad3 immunoprecipitates and *vice versa*.

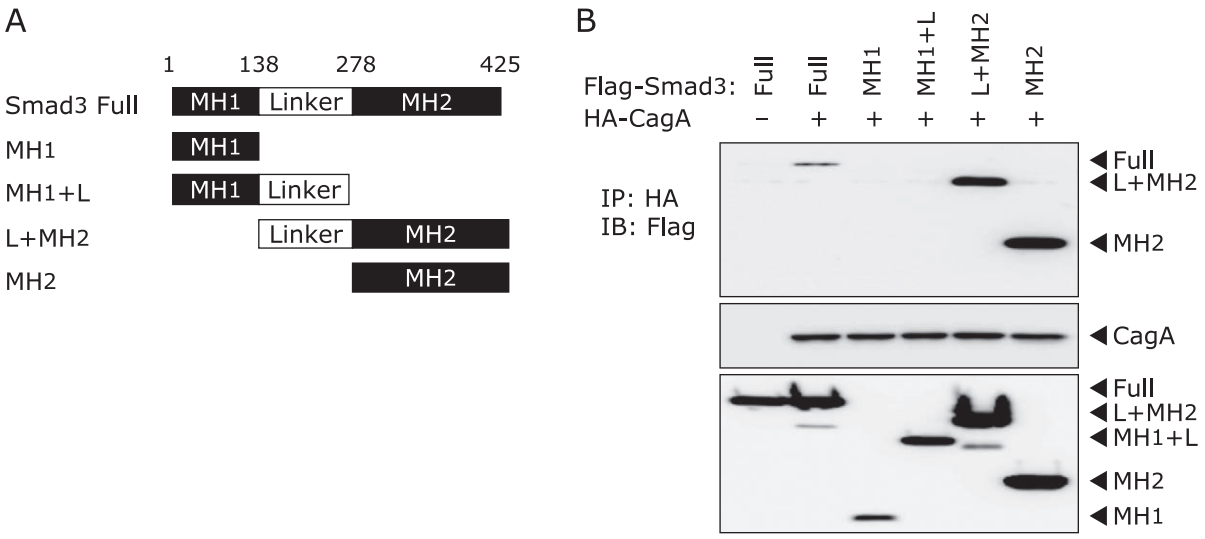

Fig. 4. *Cag*A interacts with MH2 domain of Smad3. (A) Schematic drawings of Smad3 truncation mutants. (B) HEK293T cells were transfected with Flag-Smad3 deletion mutants and with HA-CagA. Cell lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-Flag antibody.

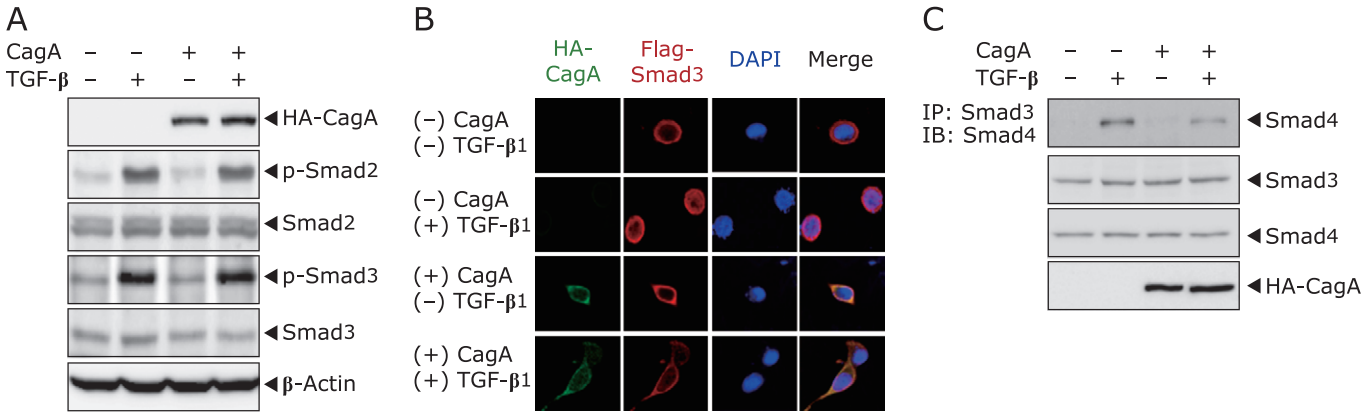

Fig. 5. CagA inhibits TGF-β-induced nuclear translocation of Smad3 and formation of Smad3-Smad4 complexes. (A) AGS cells transfected with HA-tagged CagA were treated with TGF-β1 for 1 h. Cell lysates were analyzed by immunoblotting. (B) AGS cells were transfected with Flag-Smad3 and HA-CagA, and then treated TGF-β1 for 1 h. Cells were fixed in 4% PFA, and then stained with anti-Flag or anti-HA antibody followed by Alexa Fluor goat anti-mouse or anti-rabbit IgG. (C) AGS cells were transfected with HA-CagA, and treated TGF-β1 for 1 h. Cell lysates were subjected to immunoprecipitation using anti-Smad3 antibody, followed by immunoblotting with anti-Smad4 antibody.

interaction might be responsible for repressed cancer preventive TGF-β, rendering *H. pylori*-associated gastritis and *H. pylori*-induced carcinogenesis.

Discussion

Current study clearly showed that CagA (+) *H. pylori* is responsible for propagating gastric inflammation and gastric carcinogenesis through repressed anti-inflammatory and cancer suppressive action of TGF-β, in which CagA-interacted Smads led to lowered TGF-β signaling at transcription level. Schematic summary (Fig. 6C) was presented to explain repressed TGF-β, which is pivotal cancer-suppressive cytokine, through Smads interaction with *H. pylori* CagA, can be responsible for *H. pylori*-associated gastritis as well as gastric cancer. We used the SBE4-Luc reporter construct, which contains four tandem repeats CAGACA sequence of Smad-binding element (SBE) and 3TP-Lux reporter construct, which contains three consecutive activator protein-1 (AP-1)-binding elements.[33] Since they are very specific for checking the activity of the TGF-β-dependent Smad pathway, we have no doubt to assume that with *H. pylori* CagA has an important function in repressing TGF-β signaling.

It is well known that when *H. pylori* infected into the host cells, they can suppress gastric mucosal TGF-β1.[29,30] However, the mechanism which explains clearly how *H. pylori* down-regulated TGF-β or its signaling is still not clear. Thus, we might be the first group to provide a mechanistic explanation about this issue. Moreover, we also examined the activin response element (ARE)-Luc reporter construct luciferase activity along with FAST-1 which is one of other TGF-β-sensitive reporter gene and BMP response element (BRE)-Luc reporter construct luciferase activity which is specific for BMP-2 and BMP-4. Our study showed *H. pylori* CagA only suppressed the ARE-Luc activity, suggesting that *H. pylori* CagA is very particular for TGF-β signaling not for BMP signaling. Even though we repeated our experiment in other gastric cell lines, MKN28, MKN45, and RGM-1, we could get the same result. The reason why we used AGS cells for the main experiment was that AGS cells are not only very specific for expression of transiently transfected HA-CagA but also good response for TGF-β signaling. Specially, when CagA positive *H. pylori* are infected into human gastric epithelial adenocarcinoma (AGS) cells, the tyrosine phosphorylated CagA-PY induces rearrangement of the actin cytoskeleton lead to cell elongation (the hummingbird phenotype).[34] Importantly, in AGS cells, CagA causes G1-cell cycle arrest.[35] TGF-β is the most potent inhibitor of cell cycle progression of epithelial cells.[36]

H. pylori infection or CagA totally do not have any effect on the expression of TGF-β receptors and Smads except the expression level of TGF-β1 and Smad7 (data not shown). Though some studies showed the *H. pylori* infection is associated with increased expressions of TGF-β1, TGF-β type I receptor, Smad7 and connective tissue growth factor (CTGF) in mononuclear cells (MNCs) and some epithelial cells,[37] others showed that in SNU-16 gastric cancer cell line, the level of TGF-β1 decreased within 24 h of *H. pylori* infection and after that it recovered to normal.[30] Thus, in different cell type, TGF-β may have different expression relevant to *H. pylori* infection. The hallmark of Smad7 is its ability to inhibit the association of activated TGF-β type I receptor with the substrate Smads but Smad7 was also one of TGF-β target gene. Our data showed TGF-β can induce the transcription level of Smad7 in early time point treatment. On the other hand, *H. pylori* infection and transient transfection of CagA have effect on inducing Smad7. Therefore, upon TGF-β stimulation and CagA transfection, the expression level of Smad7 did not much change.

Our recent results showed that CagA interacts strongly with MH2 domain of Smad3 independent of TGF-β. In receptor-activated Smads (R-Smads) Smad2/3 and common partner (Co-Smad) Smad4 contain two highly conserved domains, the Mad homology (MH) 1 domain and MH2 domain, which are connected by a linker region.[38] Whereas their MH1 domains can interact with the DNA, the MH2 domains are endowed with transcriptional activation and protein-protein interaction. However, only the MH1 domain of Smad3 can interact directly with SBE sequences (CAGAGTCT) in the DNA because Smad2 contains an extra exon that encodes 30 amino acids absent in the MH1 domain of Smad3 and prevents its binding to DNA.[38–40] Moreover, the important role of Smad3 as an essential mediator of the TGF-β-induced anti-inflammatory and suppressive activities at the mucosal level emerges from studies in mice with targeted deletion of the Smad3 gene.[41] Thus there is possibility that CagA suppress the TGF-β signaling by interacting with MH2 domain of Smad3.

Our data demonstrated that CagA did not have any influence on the phosphorylation of Smad2/3 even though CagA interacts with Smads. CagA is a large complex protein with ~145 kDa size. CagA interacts with various host cellular proteins to trigger distinct signaling pathways in a tyrosine phosphorylation-dependent and -independent manner.[42,43] Obviously, CagA is capable of

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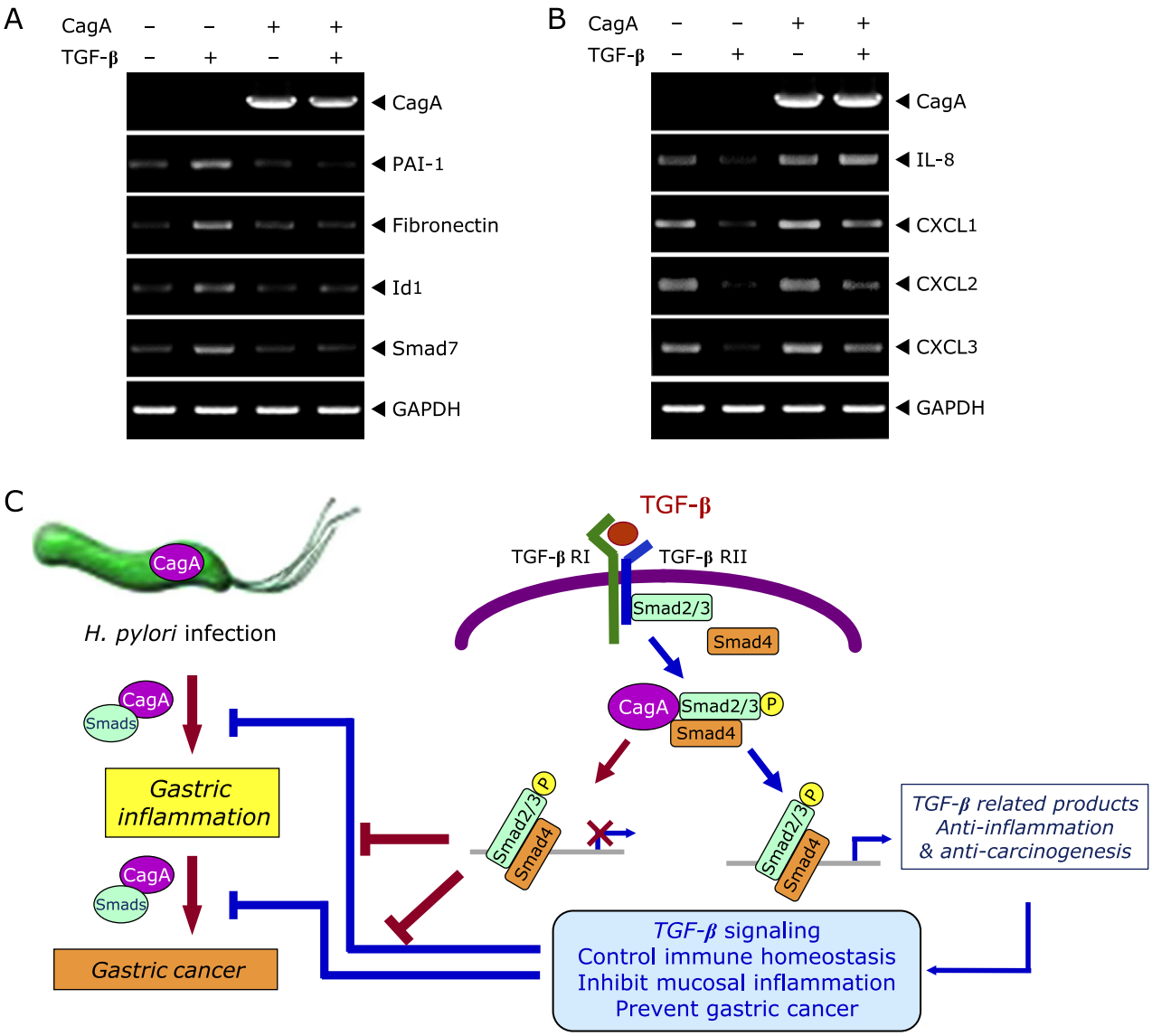

Fig. 6. CagA antagonizes TGF-β-induced expression of target genes. AGS cells were transfected with HA-CagA, and then treated TGF-β1 for 8 h. Total RNA was isolated and the mRNA expression was analyzed by RT-PCR. (A) CagA represses TGF-β-induced expression of PAI-1, fibronectin, Id1 and Smad7. (B) CagA abrogates TGF-β-induced suppression of IL-8, and chemokines CXCL1, CXCL2 and CXCL3. (C) Schematic summary to explain repressed TGF-β, which is pivotal cancer-suppressive cytokine, through Smads interaction with *H. pylori* CagA, can be responsible for *H. pylori*-associated gastritis as well as gastric cancer.

interacting with almost Smads in TGF-β signaling. However CagA strongly interact with Smad3 and reduce the endogenous complexes of Smad3 and Smad4 in the presence of TGF-β1 for 1 h. It suggests that not only Smad3 but also Smad4 has important function in the story CagA-TGF-β signaling. On the other hand, many clinical data showed that all CagA positive *H. pylori* infected biopsy specimens exhibit high levels of Smad7 compared with normal biopsy specimens and eradication of CagA positive *H. pylori* results in a dramatic inhibition of Smad7.[6,29,37]

Conclusively, in this study we clearly proved that *H. pylori* infection repressed TGF-β signaling, enabling to propagate gastric inflammation and lose cancer inhibitory action, in which lowered cancer suppressive cytokine was through *H. pylori* CagA-binding with Smads, especially with MH2 domain of Smad3. These novel findings explain the risk of gastric inflammation and gastric cancer relevant to CagA (+) *H. pylori* infection.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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