IJC
International Journal of Cancer

A positive feedback loop between STAT3 and cyclooxygenase-2 gene may contribute to *Helicobacter pylori*-associated human gastric tumorigenesis

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Persistent infection with *Helicobacter pylori* (*H. pylori*) contributes to gastric diseases including chronic gastritis and gastric cancer. However, the pathogenesis of this carcinogenic bacterium has not been completely elucidated. Here, we report that *H. pylori* rapidly triggers STAT3 signaling and induces STAT3-dependent COX-2 expression both *in vitro* and *in vivo*. STAT3 upregulats COX-2 by binding to and increasing the activity of *COX-2* promoter. COX-2 in turn regulates IL-6/STAT3 signaling under basal conditions and during *H. pylori* infection. These findings suggest that a positive feedback loop between STAT3 and COX-2 exists in the basal condition and *H. pylori* infectious condition. Immunohistochemical staining revealed that *H. pylori*-positive gastritis tissues exhibited markedly higher levels of $pSTAT3^{Tyr705}$ than *H. pylori*-negative ones. High $pSTAT3^{Tyr705}$ levels are correlated with intestinal metaplasia and dysplasia, suggesting $pSTAT3^{Tyr705}$ may be useful in the early detection of gastric tumorigenesis. Additionally, a strong positive correlation between STAT3/$pSTAT3^{Tyr705}$ levels and COX-2 expression was identified in gastritis and gastric cancer tissues. Together, these findings provide new evidence for a positive feedback loop between STAT3 signaling and COX-2 in *H. pylori* pathogenesis and may lead to new approaches for early detection and effective therapy of gastric cancer.

Carcinogenesis

Helicobacter pylori (*H. pylori*) is an important pathogen that causes a wide range of gastroduodenal diseases including chronic gastritis, peptic ulcer disease and gastric cancer.[1–3] *H. pylori* is classified as a group I biological carcinogen by the World Health Organization.[4] The primary feature of *H. pylori* infection is a chronic inflammatory infiltrate characterized by the release of proinflammatory cytokines such as cyclooxygenase-2 (COX-2), tumor necrosis factor and interleukins.[5]

Key words: cyclooxygenase-2, gastric cancer, *H. pylori*, STAT3
Additional Supporting Information may be found in the online version of this article
*H.X., W.D. and T.-T.S. contributed equally to this work.
Grant sponsor: National Basic Research Program of China 973 program Grant; **Grant number:** 2010CB5293; **Grant sponsor:** National High Technology Research and Development Program (863 Program Grant); **Grant number:** 2012AA02A504; **Grant sponsor:** NSFC; **Grant numbers:** 91129724, 30900757 and 81270480
DOI: 10.1002/ijc.28539
History: Received 26 Feb 2013; Accepted 26 Sep 2013; Online 11 Oct 2013
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Recent studies have suggested that increased COX-2 expression is an important step in *H. pylori* pathogenicity.[6] COX-2 is assumed to play a key role in *H. pylori*-associated gastric cancer and the propagation of gastric inflammation.[7] COX-2 overexpression has been detected in various cancers.[8–10] The relationship between the oncogenic mechanisms of COX-2 and *H. pylori*-induced inflammation in gastric carcinogenesis has been characterized; however, the signaling pathway involved in *H. pylori*-induced COX-2 upregulation is not yet completely understood.

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway plays an important role in numerous physiological processes. Accumulating evidence suggest that abnormalities in the JAK/STAT pathway, especially STAT3 signaling, are involved in the oncogenesis of several cancers,[11–13] including gastric cancer. STAT proteins are latent transcription factors that exist as monomers in the cytoplasm.[14,15] STATs require phosphorylation and nuclear translocation to initiate the transcription of target genes. Several inflammatory cytokines and growth factors including interleukin-6 (IL-6), interferon and epidermal growth factor trigger STAT3 activation.[2] In addition, STAT3 is activated in gastric epithelial cells infected with *H. pylori*,[2] but its role in *H. pylori* -associated gastric cancer has not been completely elucidated and the target genes of STAT3 remain unclear.

Although COX-2 and STAT3 are known to contribute to the initiation and progression of *H. pylori* -associated gastric inflammation and tumorigenesis, the crosstalk between COX-2 and STAT3 in *H. pylori* pathogenesis has yet not been identified. In this study, we evaluated the effect of *H. pylori*

What's new?
Persistent infection with *Helicobacter pylori* contributes to gastric diseases including chronic gastritis and gastric cancer. Although COX-2 and STAT3 are known to contribute to the initiation and progression of *H. pylori*-associated gastric inflammation and tumorigenesis, the crosstalk between COX-2 and STAT3 in *H. pylori* pathogenesis has yet not been identified. Here, the authors report that *H. pylori* rapidly triggers STAT3 signaling and induces STAT3-dependent COX-2 expression. COX-2 in turn regulates IL-6/STAT3 signaling. Thus, a positive feedback loop between STAT3 and COX-2 is at play in the basal condition and *H. pylori* infectious condition, and may also contribute to gastric tumorigenesis.

infection on the crosstalk regulation between STAT3 and COX-2. We identified a novel intracellular signaling network involving COX-2 and STAT3 in *H. pylori* pathogenesis. Our aim is to elucidate further the mechanisms by which *H. pylori* exerts its pathogenic effects in the human stomach.

Material and Methods

Cell culture, H. pylori infection and celecoxib treatment

In this study, we used the human gastric epithelial cell line GES-1 and three human gastric cancer epithelial cell lines: AGS (poorly differentiated), MKN-45 (poorly differentiated) and MKN-28 (well differentiated). Cells were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2.

The CagA-positive/VacA-positive NCTC11637 *H. pylori* strain was cultured with 5% sheep blood and incubated at 37°C in a microaerophilic chamber containing 10% CO_2, 5% O_2 and 85% N_2. After 72 hr, the *H. pylori* was collected and resuspended in phosphate buffered saline; a multiplicity of infection (MOI) of 100:1 was used for infection experiments. The selective COX-2 inhibitor celecoxib was purchased from Sigma-Aldrich, dissolved in dimethyl sulfoxide and stored at −20°C until use.

Western blot, antibodies and ELISA analysis of IL-6 release

Western blot analysis was performed using standard techniques. Briefly, the cells were lysed in M-PER Reagent (Pierce) to prepare whole cell lysates. And NE-PER nuclear and cytoplasmic extraction reagent kit (Pierce) was used to separate cytoplasmic and nuclear protein fractions. Equal amounts of protein (50–100 μg/lane) were separated and transferred to nitrocellulose. Proteins were detected with the enhanced chemiluminescence detection kit (SuperSignal West Femto Substrate, Pierce). The membrane was probed with monoclonal antibodies against glyceraldehyde 3-phosphate dehydrogenase as a loading control for whole cell lysates and cytoplasmic fraction and with the TATA binding protein as a loading control for the nuclear fraction. The concentrations of IL-6 in the medium were determined using an ELISA kit (R&D, Minneapolis, MN) according to the manufacturer's instruction.

Confocal microscopy

Cells were plated into 4-well chamber slides and infected with *H. pylori*, then were fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100 and blocked in 1% BSA in PBS. The cells were probed with STAT3 or $pSTAT3^{Tyr705}$ antibodies (Cell Signaling Technology) for 4 hr at 37°C and Secondary antibodies (Alexa488-anti-goat and Alexa546-anti-rabbit) were used to label STAT3 and $pSTAT3^{Tyr705}$, respectively. Afterwards the slides were mounted in DAPI Fluoromount-G (SouthernBiotech, Birmingham) and images were captured using a laser-scanning confocal microscope (LSM-710; Zeiss, Germany).

Quantitative real-time PCR analysis of STAT3 and COX-2

Quantitative real-time PCR was performed with FastStart SYBR Green Master (Roche) in the Prism 7900HT sequence detection system (Applied Biosystems). The PCR primers were: *STAT3*, 5′-GCTTTTGTCAGCGATGGAGT-3′ (forward), 5′-ATTTGTTGACGGGTCTGAAGTT-3′ (reverse) and *COX-2*, 5′-CCTGTGCCTGATGATTGC-3′ (forward), 5′-AAGTGCTGGGCAAAGAAT-3′ (reverse). PCR cycling parameters consisted of initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Each reaction was performed in triplicate and normalized to 18S RNA. Relative expression of the target genes were determined using the $2^{-\Delta\Delta Ct}$ method.[16] Thereafter, expression was expressed as fold difference relative to that of the untreated control cells.

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was carried out in normal gastric epithelial GES-1 cells and poorly differentiated gastric cancer AGS cells using a chromatin immunoprecipitation assay kit (Upstate, Millipore). Immunoprecipitated DNA was amplified with using primers specific to the *COX-2* promoter: *COX-2*-a (−774 to −565 nucleotides, containing three STAT3 binding sites [TTN4-6AA], 5′-TTTACCTTTCCCGCCTCT-3′ (forward), 5′-GACGCTTAAT AGGCTGTAT-3′ (reverse); and *COX-2*-b (−188 to 3 nucleotides, containing three STAT3 binding sites, 5′-GCTTCC TGGGTTTCCGATTT-3′ (forward), 5′-CATGCTAACCGA GAGAAC CTT-3′ (reverse). Nonspecific rabbit IgG was used as a negative control for immunoprecipitation. PCR products were resolved by 1.5% agarose gel electrophoresis. Furthermore, ChIP-quantitative real-time PCR was carried out in duplicate in a 10 μl reaction volume by using 3 μl of the eluted immunoprecipitated DNA and analysis was performed using the Prism 7900HT sequence detection system.

Enrichment was determined based on critical threshold (CT) measurements. The amount of DNA coprecipitated with specific antibody was calculated in comparison to the total input DNA used for each immunoprecipitation.

Small interfering RNA, plasmid constructs, cell transfection and luciferase assay

Small interfering RNAs (siRNAs) were obtained from Dharmacon (USA) and used to target human STAT3 (GenBank accession number NM_003150) and human COX-2 (GenBank accession number NM_000963). Cells were transfected with siRNA (50 nM) using the Dharma*FECT* 1 siRNA transfection reagent. Nonspecific siRNA was used as a negative control.

The DNA fragment encoding *STAT3* was amplified from human cDNA with the following primers: *STAT3*-sense: 5′-GCAAGCTTATGGCCCAATGGAATCAGCTACAG-3′ (the introduced *HindIII* site is underlined) and *STAT3*-antisense: 5′-GCCTCGAGCATGGGGGAGGTAG CGCACTCCG-3′ (the introduced *XhoI* site is underlined). The amplified cDNA fragment was cloned into the pGEM®-T Easy Vector (Promega), verified by sequencing and then subcloned into pcDNA3.1 to obtain the STAT3 expression plasmid (pcDNA3.1-STAT3). For transfection, the vector (5 μg) was transfected into 2×10^6 cells using Lipofectamine^{TM} 2000 (Invitrogen).

The luciferase assay was also performed in GES-1 and AGS cells. *COX-2* promoter regions (−395 to 72 nt) containing three STAT3 binding sites were amplified from genomic DNA with the primers: 5′-GGGGTACCGAAGCCAAGTG TCC-3′ (forward) and 5′-GAAGATCTGTGGGAACAGCA AG-3′ (reverse). The 467-bp PCR product was gel purified and cloned into the pGL3-Basic luciferase reporter vector (Promega) to generate the *COX-2* reporter construct (pGL3-COX2). After confirming the construct by sequence analysis, pGL3-COX2 (2 μg) was mixed with the *Renilla* control (pRL, 0.1 μg) and transfected into 1×10^5 cells using Lipofectamine^{TM} 2000. After 24 hr, the cells were washed, lysed and evaluated using the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was determined by normalizing firefly luciferase activity with that of the *Renilla* control.

In vivo infection of mongolian gerbils

Specific-pathogen-free male Mongolian gerbils (8-week-old, Zhejiang Academy of Medical Sciences, China) were used in this study. In *H. pylori* challenge study, gerbils were orogastrically challenged with suspensions of NCTC11637 (5×10^8 CFU/ml, 15 ml/kg) after overnight fasting, while control sham-infected animals were given sterile Brucella broth for 3 times at 1 day interval. At 8 wk after the bacterial inoculation, gerbils were sacrificed and gastric mucosal samples were taken for biopsy rapid urease test, microbial culture and histology. *H. pylori* status was defined as positive if all the three methods gave positive results.[17,18] A negative *H. pylori* status was considered if all tests gave concordant negative results. The remaining stomach samples were collected for immunofluorescence analysis, which was performed on paraffin-embedded gastric tissues by using antibodies against STAT3 (1:200), pSTAT3^{Tyr705} (1:50) and COX-2 (1:50). Additionally, for co-staining of COX-2 and pSTAT3^{Tyr705}, secondary antibodies (Alexa 488-anti-goat and Alexa 546-anti-rabbit) were used, respectively. Immunofluorescence analyses were performed.

In STAT3 adenovirus challenge study, two pAVsiRNA1.1-STAT3 adenoviruses, which express STAT3 shRNAs (TargetSeq CCAACAAUCCCAAGAAUGU and TargetSeq CAACAUGUCAUUUGCUGAA, Sunbio Medical Biotechnology, China) were used to knock down the expression of STAT3 in the stomach. In our study, a total of 5×10^8 plaque-forming units (pfu)[19] of pAVsiRNA1.1-STAT3 adenoviruses or pAVsiRNA1.1-negative control adenoviruses, which expresses negative control shRNA (TargetSeq TTCTCCGAACGTGTCACGT, Sunbio Medical Biotechnology, China) were orogastrically infected gerbils 4 times for 2 wk. At 2 wk after inoculation, stomach samples were collected for STAT3, pSTAT3^{Tyr705} and COX-2 detection.

To assess the role of STAT3 in *H. pylori* pathogenesis *in vivo*, gerbils were orogastrically infected with STAT3 shRNAs or control shRNA adenoviruses first. After 2 wk adenoviruses infection, then *H. pylori* was inoculated as mentioned above. Subsequently, stomach tissues were collected for gene expression detection by immunofluorescence analysis.

Patient specimens and immunohistochemical staining

Human gastric mucosa tissues were collected from 57 patients with chronic gastritis. None of the patients had taken nonsteroidal anti-inflammatory drugs, H2 receptor antagonists, proton pump inhibitors, antimicrobials or bismuth compounds in the 4 wk prior to the study. Meanwhile, archival specimens from 67 patients with histologically confirmed gastric cancer were also obtained. None of the patients received preoperative treatments such as radiotherapy or chemotherapy. The tissue sections were deparaffinized in xylene and rehydrated with a graded series of ethanol. A three-step streptavidin-biotin-horseradish peroxidase method was used and STAT3, pSTAT3^{Tyr705} and COX-2 were assessed with primary antibodies against STAT3 (1:200), pSTAT3^{Tyr705} (1:50) and COX-2 (1:50) using the LSAB+ kit (DakoCytomation, Denmark).

The slides were examined independently by two investigators. Protein expression was quantified by a visual grading system based on the extent of staining (percent of positive tumor cells graded on a scale of 0 to 4: 0, none; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, >75%) and staining intensity (graded on a scale of 0 to 3: 0, no staining; 1, wk staining; 2, moderate staining; 3, strong staining). For further analysis, we used a product of the grades of the extent and intensity of staining to define the cutoff value to classify protein expression as high (grade 12–4) or low (grade 0–3).[20] Both evaluation

methods showed comparable results. Therefore, we presented the extent of staining, which was classified into high (grades 2–4) and low (grades 0 and 1) expression, for all analyses for clarity of data presentation.

Ethics statement

All animal experiments described in this article have been conducted according to China guidelines for animal experimentation and approved by the Institutional Animal Care Committee of Shanghai Jiao-Tong University School of Medicine Renji Hospital, Shanghai, China.

All patients provided written informed consent before enrollment, the study protocol was approved by the Ethic Committee of Shanghai Jiao-Tong University School of Medicine Renji Hospital and the study was carried out according to the provisions of the Helsinki Declaration of 1975.

Statistical analysis

Statistical analyses were performed using SPSS 13.0 software (SPSS, Chicago, IL). Results were expressed as the mean ± standard deviation (SD). The data were analyzed for significance by ANOVA. The association between two categorical variables was evaluated by Fisher's exact test or chi-square test. Correlation analysis was performed between STAT3/p-STAT3^{Tyr705} and COX-2. Results were considered significant if the p-value was 0.05.

Results

H. pylori induces rapid nuclear translocation of pSTAT3^{Tyr705} and activation of STAT3 signaling

STAT3 is activated through the phosphorylation of tyrosine at residue 705, which induces dimerization, nuclear translocation and DNA binding. Thus, we detected the expression of pSTAT3^{Tyr705}, which is an essential protein for the activation of STAT3 signaling. In this study, cytoplasmic and nuclear protein fractions were separated and the ability of *H. pylori* to activate STAT3 signaling in cells was evaluated by Western blot. As shown in Figure 1*a*, nuclear levels of phosphorylated STAT3 (pSTAT3^{Tyr705}) were markedly increased and cytoplasmic levels were significantly decreased in all four cell lines 5 min after *H. pylori* infection, while total STAT3 nuclear and cytoplasmic levels appeared to be unchanged. These changes were also noted 10 min after *H. pylori* infection in these cell lines. Moreover, confocal microscopic images of STAT3/pSTAT3^{Tyr705}/DAPI in GES-1 and AGS cells showed that nuclear levels of pSTAT3^{Tyr705} were increased at 10 min after *H. pylori* infection (Fig. 1*b*). Thus, all the data indicate that activated STAT3 (pSTAT3^{Tyr705}) is immediately translocated from the cytoplasm to the nucleus after *H. pylori* infection, suggesting that *H. pylori* activates STAT3 signaling within 5 min after infection.

We then used normal human gastric epithelial cells (GES-1) and poorly differentiated gastric cancer epithelial cells (AGS) for further study. Western blot analysis revealed that levels of STAT3 and pSTAT3^{Tyr705} increased significantly

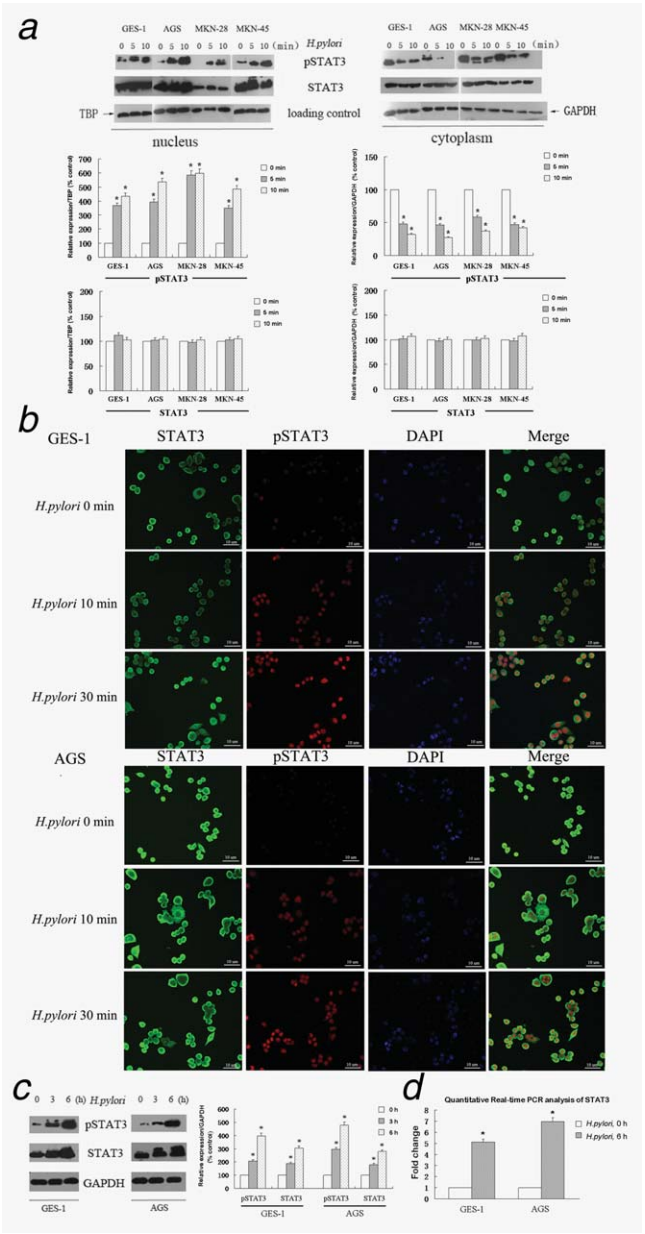

Figure 1. *H. pylori* induces STAT3 signaling. Infection experiments were conducted with MOI of 100:1. (*a*) A representative Western blot and the summarized data (below) showing that *H. pylori* induced nuclear translocation of pSTAT3^{Tyr705} within 5 min in all four cell lines without altering total STAT3 levels, suggesting that *H. pylori* induces STAT3 signaling. A similar pattern was observed 10 min after *H. pylori* infection. (*b*) Confocal microscopic images of STAT3/p-STAT3^{Tyr705}/DAPI. At 10 min after *H. pylori* infection, nuclear levels of pSTAT3^{Tyr705} (red) were increased. These changes were also noted 30 min after *H. pylori* infection. STAT3 (green), pSTAT3^{Tyr705} (red) and DAPI (blue). (*c*) A representative Western blot and the summarized data (right) showing that STAT3 and pSTAT3^{Tyr705} levels increased over time in GES-1 and AGS cells after *H. pylori* infection, consistent with activation of STAT3 signaling by *H. pylori*. (*d*) Quantitative real-time PCR analysis demonstrated that STAT3 mRNA was elevated in GES-1 and AGS cells 6 h after *H. pylori* infection. The data shown are representative of three separate experiments. Results are expressed as relative expression compared with uninfected cells (*$p < 0.05$). Each value is the mean ± SD of three experiments.

over time in both cell lines, peaking 6 hr after *H. pylori* infection (Fig. 1*c*). Real-time PCR analysis showed that STAT3 mRNA was elevated 6 hr after *H. pylori* infection, suggesting that *H. pylori* increases STAT3 expression by inducing transcription (Fig. 1*d*).

H. pylori-induced COX-2 upregulation is STAT3-dependent

We then assessed the effect of *H. pylori* infection on COX-2 expression. Western blot revealed that COX-2 protein levels were increased in GES-1 and AGS cells 6 hr after *H. pylori* infection (Fig. 2*a*). Quantitative real-time PCR analysis showed COX-2 mRNA was also elevated (Fig. 2*b*). Consistent with the elevated protein and transcript levels, the luciferase assay showed that the *COX-2* promoter was activated by *H. pylori* infection in both cells (Fig. 2*c*). Taken together, these findings demonstrate that *H. pylori* triggers COX-2 upregulation by modulation the transcription of *COX-2* promoter.

Since STAT3 is a transcription factor and is activated by *H. pylori*, we assessed the role of STAT3 in *H. pylori*-induced COX-2 upregulation. GES-1 and AGS cells were transfected with STAT3 siRNA or nonspecific (control) siRNA for 48 hr and then infected with *H. pylori* for another 6 hr. As shown in Fig. 2*d*, STAT3 knockdown blocked the *H. pylori*-induced increase in COX-2 expression. Similarly, the luciferase assay showed that *COX-2* promoter activity was stimulated by *H. pylori* (Fig. 2*e*). However, the *H. pylori*-induced *COX-2* promoter activity was dramatically inhibited by STAT3 siRNA. Therefore, these results demonstrate that *H. pylori*-induced COX-2 expression is STAT3-dependent.

To determine whether *H. pylori* induced STAT3 activation and COX-2 upregulation *in vivo*, gerbils were infected with *H. pylori* or Brucella broth and sacrificed at 8 wk after inoculation. Our immunofluorescence data showed that gastric tissue obtained from sham-infected animals displayed minimal STAT3, pSTAT3^{Tyr705} and COX-2. However, gastric tissue obtained from gerbils infected with NCTC11637 displayed increased STAT3, pSTAT3^{Tyr705}and COX-2 (Figs. 2*f* and 2*h*).

To determine whether *H. pylori* -induced COX-2 upregulation is STAT3-dependent *in vivo*, two pAVsiRNA1.1-STAT3 adenoviruses were used to knock down the expression of STAT3 in the stomach of the gerbils and then *H. pylori* were inoculated. Immunofluorescence analysis showed

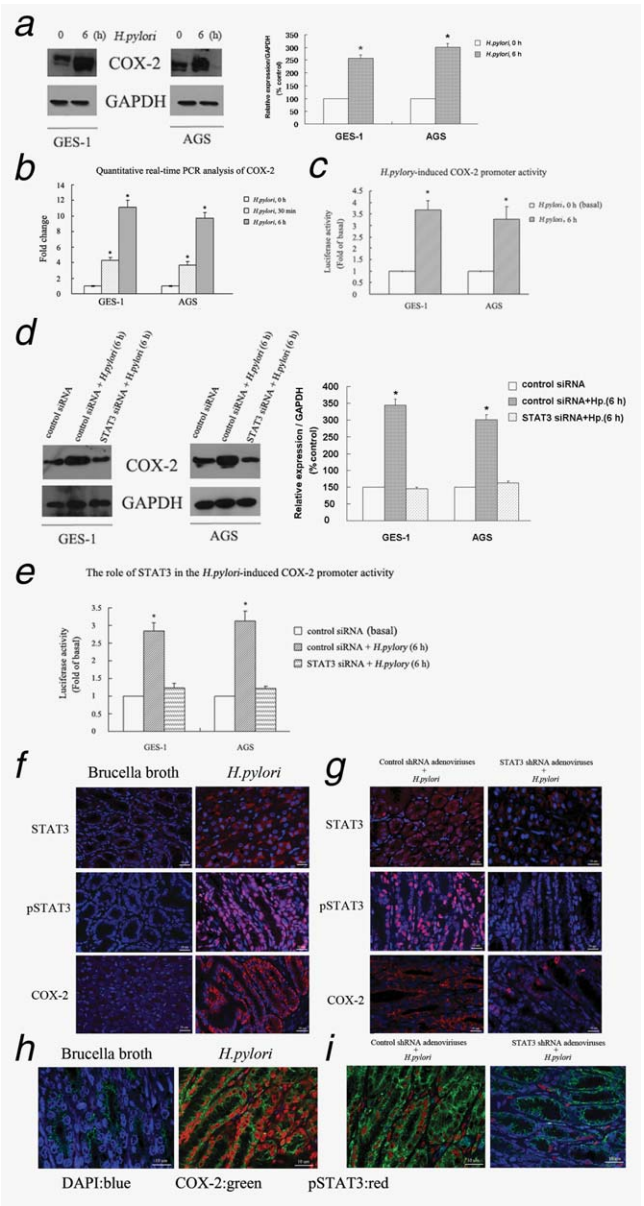

Figure 2. *H. pylori*-induced COX-2 upregulation is STAT3-dependent. A representative Western blot (*a*), the summarized data (right) and real-time PCR analysis (*b*) revealed that *H. pylori* induced upregulation of COX-2. Consistent with the elevated protein and transcript levels, the luciferase assay (*c*) showed the *COX-2* promoter to be activated by *H. pylori* infection in GES-1 and AGS cells. These data indicate that *H. pylori* triggers upregulation of COX-2 by modulation of transcription. (*d*) Western blot analysis and the summarized data (right) revealed that selectively reducing STAT3 expression by siRNA prevented *H. pylori* upregulation of COX-2 protein levels. Results of the luciferase assay (*e*) showed that *H. pylori*-induced *COX-2* promoter activity was strongly inhibited by STAT3 siRNA. These results demonstrate that *H. pylori*-induced COX-2 upregulation is STAT3-dependent. The data shown are representative of three separate experiments. Results are expressed as relative expression compared with control cells ($*p < 0.05$). Each value is the mean ± SD of three experiments. (*f*) *H. pylori* induce STAT3 activation and COX-2 upregulation *in vivo*. Gerbils were infected with *H. pylori* or Brucella broth and immunofluorescence data showed that STAT3, pSTAT3^{Tyr705}and COX-2 were higher in gastric tissue obtained from *H. pylori*-infected gerbils. G: *H. pylori* -induced COX-2 upregulation is STAT3-dependent *in vivo*. Gerbils were orogastrically infected with STAT3 shRNA or control shRNA adenoviruses first. After 2 week adenoviruses infection, *H. pylori* was inoculated. Immunofluorescence analysis showed that STAT3 knockdown blocked the *H. pylori*-induced increase in COX-2 expression *in vivo*. (*h*) Costaining of COX-2 and pSTAT3^{Tyr705} in *H. pylori* challenge study. COX-2 (green), pSTAT3^{Tyr705} (red) and DAPI (blue). (*i*) Costaining of COX-2 and pSTAT3^{Tyr705} in STAT3 adenovirus challenge study. COX-2 (green), pSTAT3^{Tyr705} (red) and DAPI (blue).

STAT3 knockdown blocked the *H. pylori*-induced increase in COX-2 expression *in vivo* (Figs. 2*g* and 2*i*). In addition, we identified a positive correlation between pSTAT3^{Tyr705} and COX-2 levels in gerbils' gastric tissues (Spearman's correlation coefficient, $r = 0.816$, $P = 0.001$). Therefore, these findings indicate that *H. pylori*-induced COX-2 expression is STAT3-dependent in gerbils.

STAT3 upregulates the protein expression and transcriptional activity of COX-2

We next addressed the mechanism that STAT3 induces COX-2 expression. As shown in Figure 3*a*, STAT3 siRNA decreased COX-2 expression and transfection of the STAT3 expression vector increased COX-2 protein levels. Thus, STAT3 is involved in upregulating COX-2 protein expression in both gastric epithelial cells and gastric cancer cells.

Moreover, DNA sequence analysis of the *COX-2* promoter (−800–0 nt) revealed seven STAT3 binding sites (Fig. 3*b*). For the ChIP assay, we designed two primer sets (*COX-2*-a, −774 to −565 nt, containing three STAT3 binding sites; *COX-2*-b, −188 to 3 nt, containing three STAT3 binding sites) to amplify ~200-bp regions of the *COX-2* promoter. As shown in Figure 3*c*, both primer sets amplified their target fragments, indicating that STAT3 binds to the *COX-2* promoter. In addition, to provide the direct evidence that *H. pylori*-induced STAT3 binds *COX-2* promoter, ChIP-quantitative real-time PCR was performed in GES-1 cells. As shown in Figure 3*d*, after 6 hr infection, *H. pylori* significantly increases the STAT3 binding to both *COX-2* promoter region, indicating that *H. pylori* induces the binding of STAT3 on *COX-2* promoter.

We also found evidence that STAT3 regulates COX-2 transcription. Results of the luciferase assay show that siRNA-mediated downregulation of STAT3 significantly reduced COX-2 transcription in GES-1 cells. Conversely, STAT3 upregulation significantly increased COX-2 transcription in GES-1 cells (Fig. 3*e*). Taken together, our data demonstrate that STAT3 upregulates COX-2 transcription.

COX-2 depletion inhibits IL-6/STAT3 signaling and COX-2 siRNA blocks *H. pylori* -induced IL-6/STAT3 signaling

Because COX-2 plays a central role in the inflammatory changes associated with the chronic *H. pylori* infection and is involved in gastric tumorigenesis,[21,22] we first assessed whether the selective COX-2 inhibitor celecoxib can inhibit STAT3 signaling. As shown in Figure 4*a*, celecoxib decreased COX-2 and total STAT3 levels in both cell lines, but this effect was not dose-dependent. In contrast, pSTAT3^{Tyr705} levels decreased with increasing concentrations of celecoxib, indicating that the decrease in pSTAT3^{Tyr705} is independent of the decrease in total STAT3. Therefore, our data suggest that celecoxib suppresses STAT3 expression and activation.

To further confirm this conclusion, we introduced COX-2 siRNA into GES-1 and AGS cells and detected the changes of the IL-6/STAT3 signaling. Our summarized data showed that COX-2 levels were successfully decreased after transfection with COX-2 siRNA for 72 hr. Simultaneously, IL-6, STAT3 and pSTAT3^{Tyr705} levels were reduced after knockdown of COX2 expression (Fig. 4*b*). Therefore, our findings suggest that COX-2 contributes to the modulation of IL-6/STAT3 signaling at the basal condition in normal human gastric epithelial cells and gastric cancer cells.

During *H. pylori* infection, GES-1 and AGS cells were transfected with COX-2 siRNA or nonspecific siRNA for 48 hr and then infected with *H. pylori* for another 6 hr. As shown in Figure 4*c*, COX-2 siRNA strongly inhibited *H. pylori*-induced upregulation of IL-6, STAT3 and pSTAT3-^{Tyr705}, suggesting the involvement of COX-2 in *H. pylori*-induced IL-6-STAT3 signaling. Taken together, our findings suggest that COX-2 modulates IL-6-STAT3 signaling under basal conditions and during *H. pylori* infection.

Activated STAT3 correlates with intestinal metaplasia and dysplasia in gastritis tissues

Immunohistochemical staining showed that STAT3 and COX-2 were detected primarily in the cytoplasm, whereas pSTAT3^{Tyr705} was found primarily in the nucleus in gastritis mucosa tissues (Fig. 5*a*). Notably, high levels of pSTAT3^{Tyr705} were detected in 60.9% (14/23) of *H. pylori* positive gastritis samples, but 35.3% (12/34) of *H. pylori* negative gastritis samples. Moreover, high levels of pSTAT3^{Tyr705} were correlated with intestinal metaplasia ($p = 0.018$) and dysplasia ($p = 0.031$, Table 1). But, pSTAT3^{Tyr705} levels were not associated with other demographic or clinical characteristics such as age or gender rates. Additionally, no relationship was found between STAT3/COX-2 levels and patient characteristics ($p > 0.05$; Table 1).

Activated STAT3 and COX-2 correlate with TNM stage in gastric cancer tissues?

We also determined STAT3, pSTAT3^{Tyr705} and COX-2 levels in human gastric cancer specimens by immunohistochemistry. High STAT3 levels were detected in all gastric cancer specimens; thus, STAT3 expression was not associated with any clinical or demographic characteristics (*e.g.*, age, gender, TNM stage and tumor size). However, high pSTAT3^{Tyr705} levels were detected in 3/23 (13%) of stage I/II specimens and 20/44 (45.5%) of stage III/IV specimens. High COX-2 levels were detected in 14/23 (60.9%) of stage I/II specimens and 39/44 (88.6%) of Stage III/IV specimens. Therefore, pSTAT3^{Tyr705} and COX-2 levels are associated with TNM stage ($p = 0.014$ and $p = 0.012$, respectively), but not age, gender or tumor size (Table 2).

STAT3/pSTAT3^{Tyr705} are positively correlated with COX-2 in chronic gastritis and gastric cancer tissues

We next evaluated the relationship between STAT3 and COX-2. Consistent with studies demonstrating that these two proteins regulate each other, we identified a strong positive correlation between STAT3 and COX-2 levels in gastritis

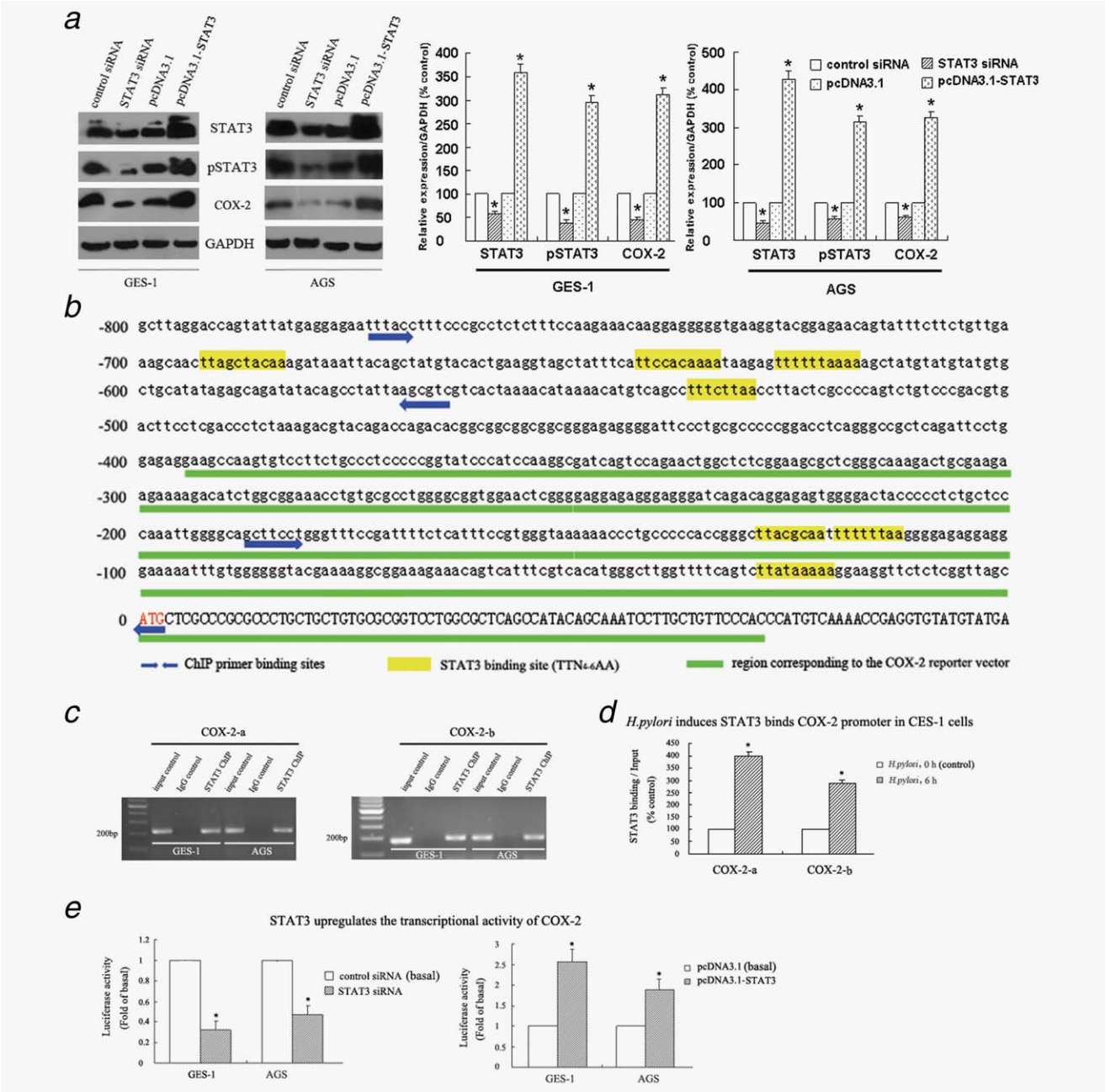

Figure 3. STAT3 upregulates COX-2 protein and mRNA expression. (*a*) Western blot analysis and the summarized data (right) showed that STAT3 siRNA decreased STAT3, pSTAT3^{Tyr705} and COX-2 expression and transfection of pcDNA3.1-STAT3 increased protein levels of STAT3, pSTAT3^{Tyr705} and COX-2. (*b*): The *COX-2* promoter map showing STAT3-binding sites, ChIP primer binding sites and the region corresponding to the COX-2 reporter vector. (*c*) GES-1 and AGS cells were evaluated by ChIP assay before (input) and after immunoprecipitation using an anti-STAT3 antibody and a nonspecific rabbit IgG antibody as the negative control. DNA binding was detected using PCR primer pairs corresponding to *COX-2* promoter regions (*COX-2*-a and *COX-2*-b). These results indicate that STAT3 directly binds to the *COX-2* promoter. (*d*) ChIP-quantitative real-time PCR was performed in GES-1 cells. After 6 h infection, *H. pylori* significantly increases the STAT3 binding to both *COX-2* promoter region, indicating that *H. pylori* induces the binding of STAT3 on *COX-2* promoter. (*e*) The luciferase assay demonstrates that siRNA-mediated downregulation of STAT3 significantly reduced COX-2 transcription. Similarly, increasing protein levels of STAT3 by pcDNA3.1-STAT3 transfection significantly increased COX-2 transcription. These results demonstrate that *H. pylori*-induced COX-2 upregulation is STAT3-dependent. Results are expressed as relative expression compared with control cells (*$p < 0.05$). Each value is the mean ± SD of three experiments.

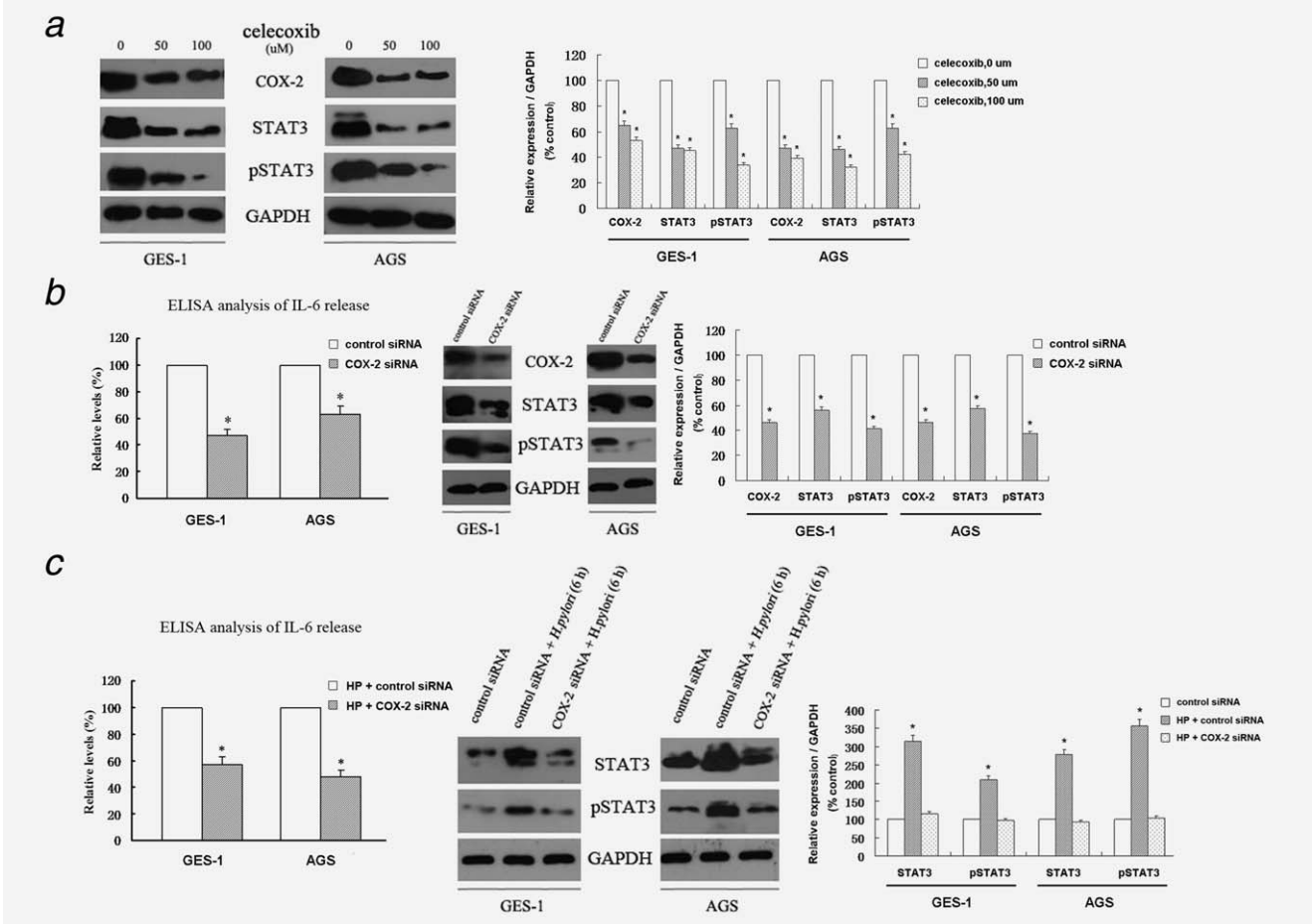

Figure 4. The role of COX-2 and celecoxib in STAT3 signaling. (*a*) Celecoxib decreased pSTAT3^{Tyr705} levels in a dose-dependent manner. Celecoxib also reduced total STAT3 and COX-2 levels, although dose-dependent patterns were not observed. These data suggest that celecoxib inhibits STAT3 signaling. The data shown are representative of three separate experiments. Results are expressed as relative expression compared with control cells (*$p < 0.05$). Each value is the mean ± SD of three experiments. (*b*) IL-6 specific ELISA and a representative Western blot showed that siRNA-mediated downregulation of COX-2 decreased IL-6, STAT3 and pSTAT3^{Tyr705}, suggesting that COX-2 modulates IL-6/STAT3 signaling under cellular basal condition. (*c*): ELISA and Western blot analysis showed that siRNA-mediated downregulation of COX-2 prevented *H. pylori* induction of IL-6, STAT3 and pSTAT3^{Tyr705}, suggesting that COX-2 mediates *H. pylori* -induced IL-6/STAT3 signaling in GES-1 and AGS cells.

(Spearman's correlation coefficient, gastritis mucosa, $r = 0.701$, $p = 0.001$) and gastric cancer ($r = 0.503$, $p = 0.001$). Similarly, pSTAT3^{Tyr705} was correlated with COX-2 in gastritis mucosa ($r = 0.643$, $p = 0.001$) and gastric cancer tissues ($r = 0.629$, $p = 0.001$; Fig. 5*b*).

Discussion

Although a great deal of progress has been made over the past two decades in understanding the pathogenesis and carcinogenesis of *H. pylori*-induced gastric disorders, the detailed mechanisms remain unclear.[23] STAT3 and COX-2 play central roles in many important cellular processes, including the inflammatory response, tumorigenesis and tumor progression. At present, the roles of COX-2 and STAT3 in *H. pylori* infection have been studied, however, their crosstalk in the development and progression of gastric disorders remain to be clarified. Here, we demonstrated that *H. pylori* activates STAT3 signaling within 5 min after infection, suggesting that STAT3 is involved in *H. pylori* pathogenesis and STAT3 activation might be the first step in the signaling of *H. pylori* -mediated inflammation and tumorigenesis.

COX-2 is upregulated in the gastric mucosa during *H. pylori* infection; however, the precise mechanism of this increase is not well understood. Lo *et al.* reported that STAT3 cooperates with both epidermal growth factor receptor (EGFR) and EGFRvIII to activate the *COX-2* gene promoter in glioblastoma,[24] but the effect of STAT3 on COX-2 expression during *H. pylori* infection is still unknown. Our results indicate that *H. pylori* -induced COX-2 upregulation is STAT3-dependent both *in vitro* and *in vivo*. Further, our data confirm that STAT3 upregulates COX-2 expression. We also analyzed the *COX-2* promoter. Results of the ChIP assay indicated that STAT3 directly binds to the *COX-2* promoter

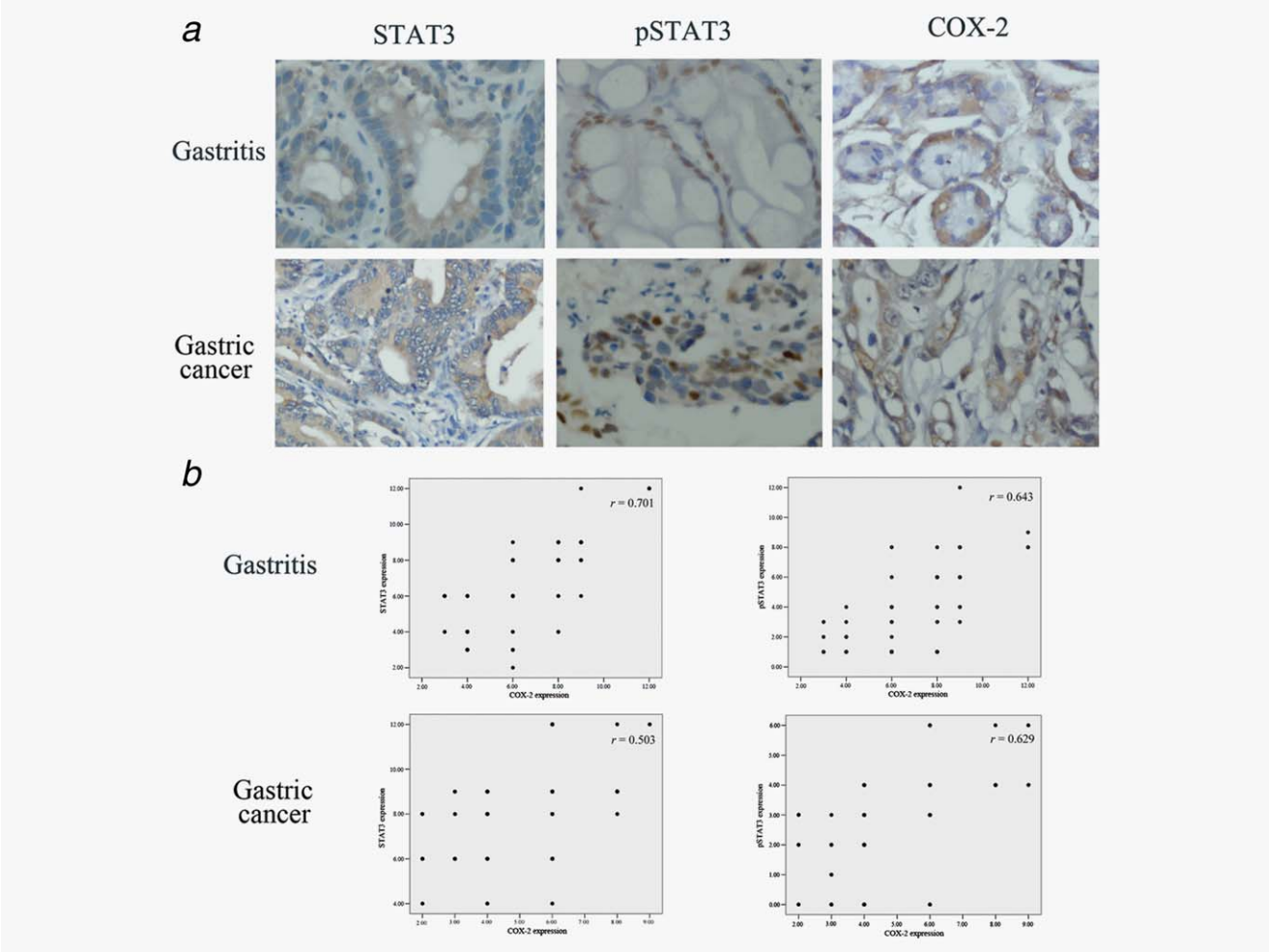

Figure 5. (*a*) Immunohistochemical staining of STAT3, pSTAT3^{Tyr705} and COX-2 in precancerous and gastric cancer lesions. STAT3 and COX-2 staining was detected primarily in the cytoplasm; however, pSTAT3^{Tyr705} was detected in the nucleus (magnification: 400×). (*b*) The scatter plot shows a strong positive correlation between STAT3/pSTAT3^{Tyr705} and COX-2 levels in precancerous and cancerous tissues.

and *H. pylori* induces the binding of STAT3 on *COX-2* promoter. The luciferase assay demonstrated that STAT3 directly activated the *COX-2* promoter. Ji Hye Seo *et al.* reported that *H. pylori* induces COX-2 expression by activating transcription factors such as nuclear factor (NF-κB).[25] Both STAT3 and NF-κB play crucial and integrated roles in inflammatory responses that promote cancer development and growth.[26–29] Moreover, both transcription factors are frequently persistently activated in the same cells and induce the expression of a highly overlapping repertoire of tumor-associated genes.[26] Nevertheless, there are differences between STAT3 and NF-κB. For example, NF-κB is involved in antitumor immune responses as well as procarcinogenic inflammation.[30,31] In contrast, STAT3 mediates tumor-promoting inflammation and suppresses antitumor immunity.[15,32] Therefore, inhibiting NF-κB may promote tumor growth and long-term blockade of NF-κB may cause substantial immune suppression.[31] However, targeting STAT3, a central regulatory node on which many oncogenic and inflammatory pathways converge, holds untapped promise for future cancer therapy.[26]

Interestingly, we found that siRNA-mediated knockdown of COX-2 decreased levels of IL-6, STAT3 and pSTAT3^{Tyr705}, indicating that COX-2 modulates IL-6/STAT3 signaling. Nevertheless, the mechanism by which COX-2 regulates IL-6 remains unclear. Further, we revealed that COX-2 is also involved in modulation of *H. pylori*-induced IL-6/STAT3 signaling. Taken together, our results suggest that STAT3 and COX-2 require each other for their persistent activation in *H. pylori*-associated inflammation, tumorigenesis and progression.

Although numerous studies have reported the importance of STAT3 in tumorigenesis and progression, the role of STAT3 in precancerous lesions is unclear. Our data show that high pSTAT3^{Tyr705} levels correlated with intestinal metaplasia and dysplasia, suggesting that STAT3 activation contributes to the malignant transformation of gastric mucosa. Thus, we speculate that dysplasia of the gastric mucosa with abnormal STAT3 activation may indicate precancerous changes; pSTAT3^{Tyr705} may be a tumorigenic factor and marker of early gastric tumorigenesis. Although

Table 1. Correlation between STAT3, pSTAT3^{Tyr705} and COX-2 levels and clinicopathologic characteristics in chronic gastritis patients

	STAT3			p-STAT3^{705}			COX-2		
	High	Low	*p* value	High	Low	*p* value	High	Low	*p* value
Sex									
Male	25	5	0.427	12	18	0.370	27	3	1.000
Female	25	2		14	13		25	2	
Age									
≤55	34	3	0.226	18	19	0.532	32	5	0.151
>55	16	4		8	12		20	0	
H. pylori									
Positive	21	2	0.689	14	9	0.057	20	3	0.384
Negative	29	5		12	22		32	2	
Intestinal metaplasia									
Yes	29	3	0.687	19	13	0.018[1]	29	3	1.000
No	21	4		7	18		23	2	
Dysplasia									
Yes	18	2	1.000	13	7	0.031[1]	19	1	0.647
No	32	5		13	24		33	4	

[1]Strong pSTAT3Tyr705 expression correlated with intestinal metaplasia and dysplasia ([1]$p < 0.05$; Fisher's exact test or chi-square test), indicating that pSTAT3Tyr705 may be useful in the early detection of gastric tumorigenesis.

Table 2. Correlation between pSTAT3^{Tyr705}, COX-2 and clinicopathologic characteristics of patients with gastric cancer

	p-STAT3^{705}			COX-2		
	High	Low	*p* value	High	Low	*p* value
Sex						
Male	13	30	0.35	34	9	1.000
Female	10	14		19	5	
Age						
≤55	14	24	0.62	30	8	0.97
>55	9	20		23	6	
TNM stage						
I/II	3	20	0.014[1]	14	9	0.012[1]
III/IV	20	24		39	5	
Tumor diameter (cm)						
≥5	12	19	0.48	25	6	0.77
>5	11	25		28	8	

[1]pSTAT3^{Tyr705} and COX-2 levels are significantly associated with TNM stage ($p = 0.014$ and $p = 0.012$, respectively, Fisher's exact test or chi-square test), suggesting that constitutive activation of STAT3 and elevation of COX-2 may lead to worse prognosis in patients with gastric cancer.

pSTAT3^{Tyr705} levels were not significantly associated with *H. pylori* infection ($p = 0.057$), *H. pylori*-positive tissues exhibited markedly higher levels of pSTAT3^{Tyr705} (60.9%) than *H. pylori*-negative samples (35.3%; Table 1). Larger sample sizes are needed to better assess this relationship. Consistent with the results of previous studies,[33–36] our data showed that constitutive activation of STAT3 and elevation of COX-2 may contribute to worse prognosis in gastric cancer patients. We also found that that STAT3/pSTAT3^{Tyr705} positively correlated with COX-2 in both gastritis and cancer tissues. These observations, together with our laboratory studies, provide evidence for a positive feedback regulation between STAT3 signaling and COX-2 in gastric inflammation and tumorigenesis.

This study demonstrated that *H. pylori* activates STAT3 signaling and induces STAT3-dependent COX-2 expression. STAT3 appears to upregulate COX-2 by binding to its promoter and enhancing *COX-2* transcription. Further, COX-2 appears to modulate STAT3 signaling, suggesting a positive feedback regulation between STAT3 and COX-2. In addition, this loop exists in both normal gastric epithelial cells and

gastric cancer cells, under basal conditions and during *H. pylori* infection. Thus, we speculate that positive feedback regulation between STAT3 and COX-2 contributes to gastric tumorigenesis (summarized in Supporting Information data). Further studies are needed to confirm our findings and to identify which STAT3 binding site is critical to COX-2 expression. Meanwhile, A more comprehensive understanding of the mechanism whereby *H. pylori*-induced STAT3 activation in short time is necessary and further studies are needed.

Acknowledgements

The funding had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Author contributions

JH and HX conceived the study and drafted the manuscript. JH, JYF and HX designed the study. WD, TTS, YWL and JLW performed experiments and collected data. All authors were involved in data analysis, data interpretation and approving final version of manuscript.

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