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Journal of Cellular Biochemistry 113:3740–3751 (2012)

Journal of Cellular Biochemistry

H. pylori Induces the Expression of Hath1 in Gastric Epithelial Cells Via Interleukin-8/STAT3 Phosphorylation While Suppressing Hes1

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ABSTRACT

Chronic gastritis associated with *Helicobacter pylori* is a leading cause of gastric intestinal metaplasia (IM), which arises from abnormal cell differentiation of the epithelium in the gastric mucosa. However, the mechanisms involved in *H. pylori*-mediated IM remain elusive. The aim of our study was to explore the effects and the underlying mechanisms of *H. pylori* on the abnormal expression of Hath1 and Sox2 and to reveal its relationship to the development of gastric IM. We found that Hath1 and Sox2 were overexpressed in gastric IM tissue. Hath1 expression was up-regulated, whereas Sox2 expression, which was independent of the CagA virulence factor, was down-regulated in gastric epithelial cells and coincided with increased IL-6 and IL-8 levels in the culture media. Stimulation with *H. pylori*-related cytokine IL-8, but not IL-6 or IL-1β, was induced by Hath1 expression in the gastric epithelial cells. Although IL-8 and IL-6 levels correlated with STAT3 (signal transducer and activator of transcription) phosphorylation before and after *H. pylori* eradication in the gastric mucosa, only the blocking of IL-8-induced STAT3 activation using AG490 or STAT3-targeting RNA interference altered Hath1 expression. Additionally, we found that *H. pylori* down-regulated Hes1, which is a direct downstream target gene of Notch signaling and a repressor of Hath1 expression. These findings suggest that *H. pylori* induced inflammation up-regulate Hath1 expression via interleukin-8/STAT3 (IL-8) phosphorylation while suppressing Hes1, which provides a novel molecular connection between a *H. pylori* infection and intestinal metaplasia. J. Cell. Biochem. 113: 3740–3751, 2012.

KEY WORDS: *H. pylori*; HATH1; SOX2; CYTOKINES; STAT3; INTESTINAL METAPLASIA

The chronic colonization of *Helicobacter pylori* in the gastric mucosa is widely known as a leading cause of atrophic gastritis and intestinal metaplasia (IM) through the chronic inflammation induced by the host immune response to *H. pylori* [Adamu et al., 2010]. The long-term inflammation is caused by many cytokines that are produced by infiltrated inflamed cells and gastric epithelial cells under the stimulation of a variety of *H. pylori* virulence factors, such as IL-8, IL-6, IFN-γ, TNF-α, IL-1β, and IL-4 [Basso et al., 2010]. However, the underlying molecular mechanisms of the host immune response to *H. pylori* that causes IM remain largely unknown.

H. pylori infection is commonly associated with IM, and there has been much interest in identifying the mechanisms behind this relationship. The aberrant differentiation of progenitor cells is one of the origins of IM. The transcription factors that regulate normal differentiation of the gastrointestinal epithelium are involved in this

The authors declare that there are no conflicts of interest.

Xin Zhang and Yongtao Yang contributed equally to this work.

Additional supporting information may be found in the online version of this article.

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 81000154, 81170340; Grant sponsor: Natural Science Foundation Project of Chongqing; Grant number: CQ CSTC 2008BA5034.

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Manuscript Received: 10 February 2012; Manuscript Accepted: 28 June 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 11 July 2012

DOI 10.1002/jcb.24248 • © 2012 Wiley Periodicals, Inc.

process. Asonuma et al. [2009] demonstrated that *H. pylori*-induced IFN-γ production led to the down-regulation of Sox2 on IL-4/STAT6 signaling, which may lead to pre-cancerous gastric atrophy and IM. The expression of the intestinal homeodomain protein CDX2 converts the stomach epithelium into an intestinal type of tissue. This finding led us to hypothesize that other intestinal stem cell differentiation regulators may be up-regulated and involved in the mucous cell metaplasia of the gastric mucosa. *Hath1*, a gene downstream of the Notch signaling pathway, is a candidate regulator that is essential for intestinal goblet cell differentiation. Previous researchers have found almost no detectable Hath1 expression in the normal gastric mucosa; in contrast, Hath1 is expressed in several types of gastric cancer cell lines, and its expression correlates with intestinal mucin production [Park et al., 2006; Yeh et al., 2009]. An in vitro analysis also demonstrated that Hath1 transcriptionally regulates mucin genes, such as MUC6 and MUC5AC, in gastric epithelial cells [Sekine et al., 2006], which is similar to Hath1 regulation of MUC2 expression in colorectum cancer cells. Moreover, we observed that there was Hath1 overexpression in the IM tissues that had become inflamed from the *H. pylori* infection. This finding suggested that the abnormal Hath1 expression participated in the development of *H. pylori*-induced IM. In fact, IM occurrence is the result of the aberrant expression of many differentiation regulation factors caused by the stimulus of many pathological factors for a long period. Therefore, inducing the IM phenotype on the normal gastric epithelial cells within a short period is challenging. In our present study, there was no Hath1 expression in the immortalized gastric epithelial cell line GES-1; thus, we abandoned the use of this non-cancer original cell line. Moreover, there is a basis for using cancer cell lines to investigate the aberrant expression of some proteins that are involved in the development of IM, such as CDX2, in which AGS, MKN45, and HCT116 cell lines have been used to explore the possible mechanisms of CDX2 expression regulation [Barros et al., 2008, 2011].

A feature of many cytokines is that they engage with a class of non-tyrosine kinase cell surface receptors that signal to the cell nucleus by way of the JAK–STAT (Janus kinase–signal transducer and activator of transcription) signaling pathway [Burger et al., 2005]. STAT3 is a member of the STAT family and is activated by numerous cytokines that are associated with the immune response to *H. pylori* [Wen et al., 2007]. Notably, IL-6 activates STAT3 through the receptor subunit gp130 [Lee et al., 2010]. The IL-6/gp130/STAT3 pathway has been demonstrated to play a role in the development of gastric cancer. Interleukin-8 (IL-8) is another proinflammatory cytokine that is associated with a *H. pylori* infection in the gastric mucosa. The increased expression of IL-8 and/or its receptors CXCR1 and CXCR2 has been observed in cancer cells, endothelial cells, infiltrating neutrophils, and tumor-associated macrophages, which suggests that IL-8 may function as a significant regulatory factor within the tumor microenvironment [Fernando et al., 2011]. Research has shown that the IL-8 expression leads to constitutive STAT3 phosphorylation on Tyr705 through CXCR2, which facilitates the transformation of NIH 3T3 cells [Burger et al., 2005]. That this phosphorylation could be blocked by the specific JAK2 inhibitor AG490 suggests that the JAK2–STAT3 pathway plays a critical role in IL-8/CXCR2-induced cell transformation [Burger et al., 2005]. Additionally, Notch downstream target genes, that is, Hes1 and Hes5, which normally suppress Hath1 expression, promote STAT3 phosphorylation by facilitating complex formations between JAK2 and STAT3 [Kamakura et al., 2004]. This body of research led us to hypothesize that cytokine-induced STAT3 phosphorylation during inflammation may up-regulate Hath1 expression, which may then induce the development of IM in the gastric mucosa.

In our study, we found that Hath1 expression was up-regulated in the gastric IM tissue. *H. pylori*-induced Hath1 expression in gastric epithelial cells was independent of the presence of the CagA gene and mediated via IL-8/STAT3 phosphorylation while suppressing Hes1. Our research proves that a novel molecular process connects a *H. pylori* infection to IM.

MATERIALS AND METHODS

H. pylori AND CELL CULTURE CONDITIONS

The ΔCagA strain (the standard isogenic mutant strain of ATCC43579), in which the CagA gene has been knocked out, was obtained from Professor Quanming Zou of the Third Military Medical University with the consent of Professor Chihiro Sasakawa of the University of Tokyo who originally constructed this mutant strain [Asahi et al., 2000]. Both standard *H. pylori* strains, that is, ATCC26695 and ΔCagA, were passaged and cultured as previously described in the literature [Zhang et al., 2011]. The GES-1, SGC7901, and HCG27 cell lines were donated by the Viral Hepatitis Research Institute of Chongqing Medical University (Chongqing, China). The BGC823, MKN45, and MKN28 cell lines were purchased from the Cancer Institute and Hospital of the Chinese Academy of Medical Sciences (Beijing, China). All of the cells were cultured in the standard manner.

TISSUE SAMPLES

Patients with dyspeptic symptoms who were scheduled for an upper gastrointestinal endoscopy were prospectively enrolled into the study. Patients were excluded if they were over 70-year old, had a malignancy, or had been treated with steroids and non-steroidal anti-inflammatory drugs, proton pump inhibitors, bismuth salts, or antibiotics within the 2 months prior to the endoscopy. The presence of a *H. pylori* infection was confirmed by at least one positive result out of the following three different methods for determining *H. pylori* status: histology, the rapid urease test, or the [^{13}C] urea breath test. The patients who were diagnosed with gastritis received a 10-day course of proton pump inhibitor-based triple therapy. The follow-up endoscopy was conducted at the end of the second month after therapy to assess for any changes in the patient's status. *H. pylori* negativity was defined using the three tests 2 months after eradication. The study was approved by the local clinical research ethics committee. All of the subjects provided informed consent before their endoscopy.

CHALLENGE OF CELLS WITH *H. pylori*

The *H. pylori* lysates and culture supernatants were prepared as previously described in the literature [Bebb et al., 2003; Zhang et al., 2011]. The MKN45 and MKN28 gastric epithelial cell lines were

co-cultured with *H. pylori* 26695 and ΔCagA *H. pylori*. For the co-culture experiments, the *H. pylori* strains were suspended in the culture medium with 1% FBS for 24 h after sub-culturing and were added to cells that were seeded to the culture plate overnight at a concentration of 20×10^6 cfu/ml. After co-culturing, the cells and the culture medium were harvested at the designated time.

CELL TREATMENT WITH CYTOKINES

MKN28 cells were seeded to the culture plate at a density of 1×10^6 cells/well overnight in the culture medium. The cells were incubated in serum-free basal medium for 6 h before treatment. The cells were then exposed to various concentrations of IL-1β, IL-6, and IL-8 at various times. For the cell signaling experiments, the cultured cells were treated with Janus kinase inhibitor tyrphostin AG490 at a 50 μM concentration 30 min prior to the addition of the cytokines.

IL-6 AND IL-8 MEASUREMENTS

The antral biopsy samples were homogenized, and aliquots of the homogenate supernatants were obtained by centrifugation and assayed for total protein by the Bradford assay (Pierce). The supernatants were diluted to 0.50 mg/ml total protein and were frozen at −80°C until assayed. IL-6 and IL-8 were measured in the human sample supernatants, and the MKN 28 culture supernatant was stimulated with *H. pylori* using a sensitive ELISA kit (R&D Systems) according to the manufacturer's instructions.

IMMUNOHISTOCHEMISTRY

A hematoxylin and eosin staining method was used to analyze the gastritis and Giemsa was used to detect *H. pylori*. The immunohistochemistry staining was performed as previously described in the literature [Yang et al., 2010]. The primary antibodies used were rabbit anti-Sox2 (1:400, Millipore), rabbit anti-Hath1 (1:400, Chemicon), and rabbit anti-phospho-Stat3 (Tyr705) (1:150, Cell Signaling). All of the primary antibodies were detected with a ready-to-use biotinylated anti-rabbit secondary antibody (Zhongshan Company, Beijing, China).

IMMUNOBLOTTING

Immunoblotting was performed as previously described in the literature [Yang et al., 2010]. The membranes were incubated overnight at 4°C in a blotting solution with anti-Hath1 (1:1,000, Chemicon), anti-Sox2 (1:1,000, Millipore), anti-Hes1 (1:1,000, Millipore), anti-STAT3 (1:1,000, Cell Signaling), anti-phospho-STAT3 (1:1,000, Cell Signaling), and anti-GAPDH (1:500, Zhongshan Company) antibodies. The blotting bands of the proteins of interest and GAPDH were scanned and semi-quantitated according to their identities; three independent experiments were performed. The protein/GAPDH ratios of the immunoreactive area were calculated by densitometry for further statistical analysis.

REPORTER ASSAY

MKN28 cells were co-transfected with 1 μg of a reporter construct (pGa981-6) and 50 ng of the *Renilla* luciferase reporter plasmid (pRL-TK, Promega) according to the manufacturer's instructions (Invitrogen). After 6 h of transfection, the cells were challenged with the *H. pylori* 26659 and ΔCagA strains at a concentration of 20×10^5 cfu/ml or with IL-6 and IL-8 at a concentration of 50 ng/ml. After stimulation for 24 h, the firefly and *Renilla* luciferase activities were measured using a TD-20/20 luminometer (Turner Designs, CA).

RNA INTERFERENCE

RNA interference was performed as previously described in the literature [Yang et al., 2010]. Briefly, STAT3 was targeted for RNAi (shRNA-STAT3) using the following sequences: 5′-GATCCTAACTT-CAGACCCGTCAACAAATTCAAGAGATTTGTTGACGGGTCTGAAG-TTTTTTTTCA-3′ and 5′-AGCTTGAAAAAAAACTTCAGACCCGT-CAACAAATCTCTTGAATTTGTTGACGGGTCTGAAGTTAG-3′. The sequences were cloned into pRNAT-U6.1 that had been digested with *Bam*HI and *Hin*dIII. In this study, the control plasmid pRNAT-U6.1-shRNA-Ctr that was had been previously constructed [Yang et al., 2010]. The MKN28 cells were transfected with pRNAT-U6.1-shRNA-STAT3 or pRNAT-U6.1-shRNA-Ctr. The transfected cells were selected using G418 (400 μg/ml, Invitrogen). Cell clones in the presence of G418 were isolated and used to build stable transfected cell lines.

STATISTICAL CALCULATIONS

For continuous variables, the data were expressed as the mean ± standard deviation. Differences between groups of band densities, cytokine levels, and Notch activity were estimated by Student's *t*-test and repeated measures ANOVA analysis. The paired *t*-test was used in the comparison of IL-6 and IL-8 levels in the gastric mucosa before and after *H. pylori* eradication. The correlation between IL-8 and IL-6 levels with STAT3 phosphorylation before and after *H. pylori* eradication was calculated by Spearman's rank correlation test. All differences were deemed significant at the level of $P < 0.05$ and very significant at the level of $P < 0.01$. Statistical analyses were performed by the SPSS 13.0 for Windows software package.

RESULTS

HATH1 AND SOX2 EXPRESSION IN THE NORMAL GASTRIC MUCOSA AND IM

We first used immunohistochemistry to investigate the changes in Hath1 and Sox2 expression levels in the normal gastric mucosa and in the IM tissue. As shown in Figure 1, Sox2 immunoreactivity was highly detectable in the nuclei of mucous cells of the neck zone of the antral glands and mucous cells in the crypt area of the normal gastric mucosa (A). Sox2 was strongly stained in the nuclei of mucous cells, which included goblet and non-goblet cells, and within the IM tissue (B). Hath1 was mainly expressed in the crypt area of the gastric gland and weakly expressed in the nuclei of mucous cells of the neck zone of the normal gastric gland (C). However, Hath1 was strongly expressed in the nuclei of goblet and non-goblet cells within the IM mucosa (D). These results indicated that Sox2 and Hath1 were aberrantly expressed in IM tissues, and there was a significant tendency of Hath1 to increasingly express in the IM tissues.

HATH1 AND SOX2 EXPRESSION IN GASTRIC CANCER CELL LINES

The regulation of Hath1 and Sox2 transcription factors was documented by investigating Hath1 and Sox2 expression in the

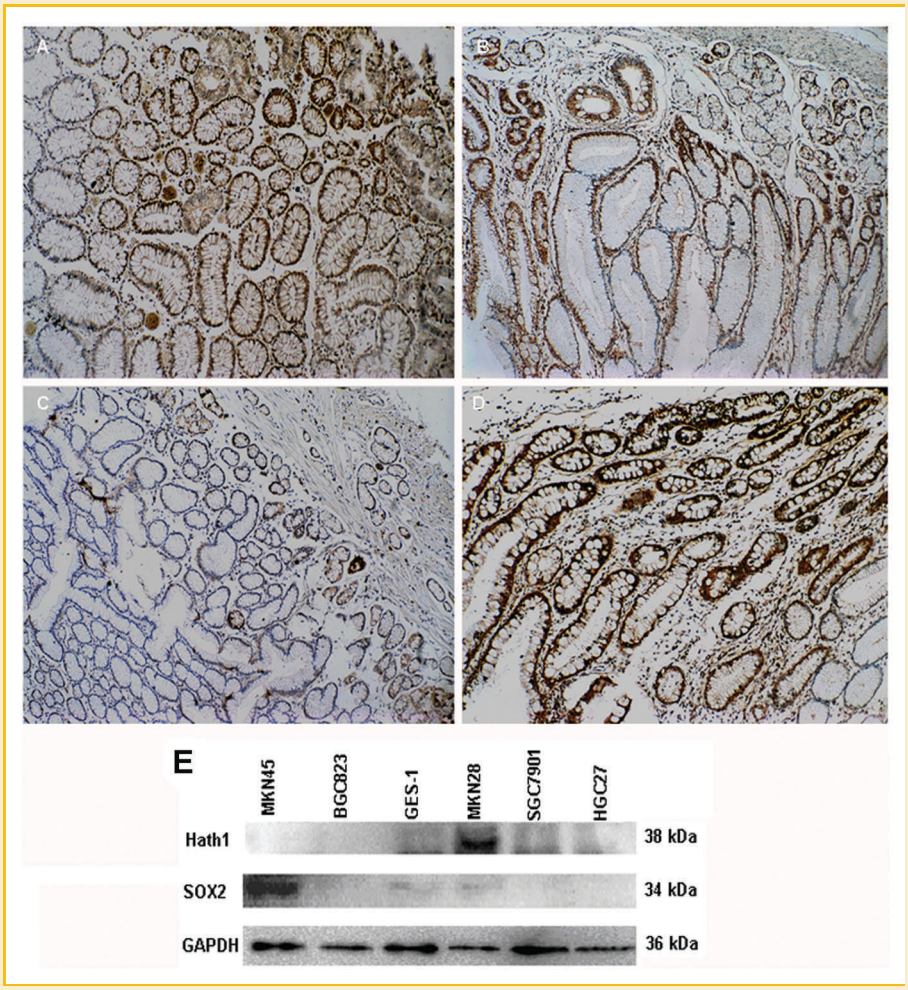

Fig. 1. Sox2 and Hath1 expression in the gastric mucosa and IM tissue. Sox2 was positively stained in the nuclei of normal gastric gland epithelial cells (A) and in intestinal metaplasia (IM) cells (B). Hath1 was weakly stained in the nuclei of normal gastric gland epithelial cells (C) but densely stained in the nuclei of IM cells (D). The lysates from six gastric cancer cell lines were immunoblotted with anti-Sox2 and anti-Hath1 antibodies. Sox2 was highly expressed in the MKN45 cell line, and Hath1 was highly expressed in the MKN28 cell line (E).

following gastric cancer cell lines: MKN45, MKN28, GES-1, BGC823, SGC7901, and HGC27. Hath1 protein was only expressed in MKN28 cells and not expressed in MKN45, GES-1, BGC823, SGC7901, and HGC27 cells. Sox2 was strongly expressed in MKN45 cells, weakly expressed in MKN28 and GES-1 cells, and not expressed in BGC823, SGC7901, and HGC27 cells (Fig. 1E). Because of these results, MKN28 and MKN45 cell lines were selected for further investigation into the regulation of Hath1 and Sox2 expression, respectively.

BOTH CAGA(−) AND CAGA(+) *H. pylori* INCREASED HATH1 EXPRESSION AND INHIBITED SOX2 EXPRESSION IN GASTRIC EPITHELIAL CELLS

To determine whether Hath1 and Sox2 expression in gastric epithelial cells would be altered by a *H. pylori* infection, cultured MKN45 and MKN28 cells were infected with a viable strain of *H. pylori* (CagA+ strain 26695; ATCC), the supernatant of the *H. pylori* culture and the sonicated *H. pylori* lysate, respectively. As shown in Figure 2, Sox2 protein levels decreased in a time-dependent manner after MKN45 cells were infected with viable CagA+ *H. pylori* (A). However, no changes in Sox2 expression were found within 24 h after stimulation with the supernatant of the *H. pylori* culture (Supplementary Fig. S1A) and the sonicated lysate of the cultured *H. pylori* (Supplementary Fig. S1B). Hath1 protein levels increased in a time-dependent manner after MKN28 cells were infected with the CagA+ *H. pylori* (Fig. 2B). However, no changes in Hath1 expression were found within 24 h after stimulation with the supernatant of the *H. pylori* culture (Supplementary Fig. S1C) and the sonicated lysate of the cultured *H. pylori* (Supplementary Fig. S1D). Because we wanted to define the effects of CagA on Hath1 and Sox2 expression, we performed a similar experiment using a CagA knockout *H. pylori* (ΔCagA *H. pylori*). Sox2 protein levels decreased in MKN45 cells (Fig. 2C), whereas Hath1 protein levels increased in MKN28 cells (Fig. 2D) in a time-dependent manner after

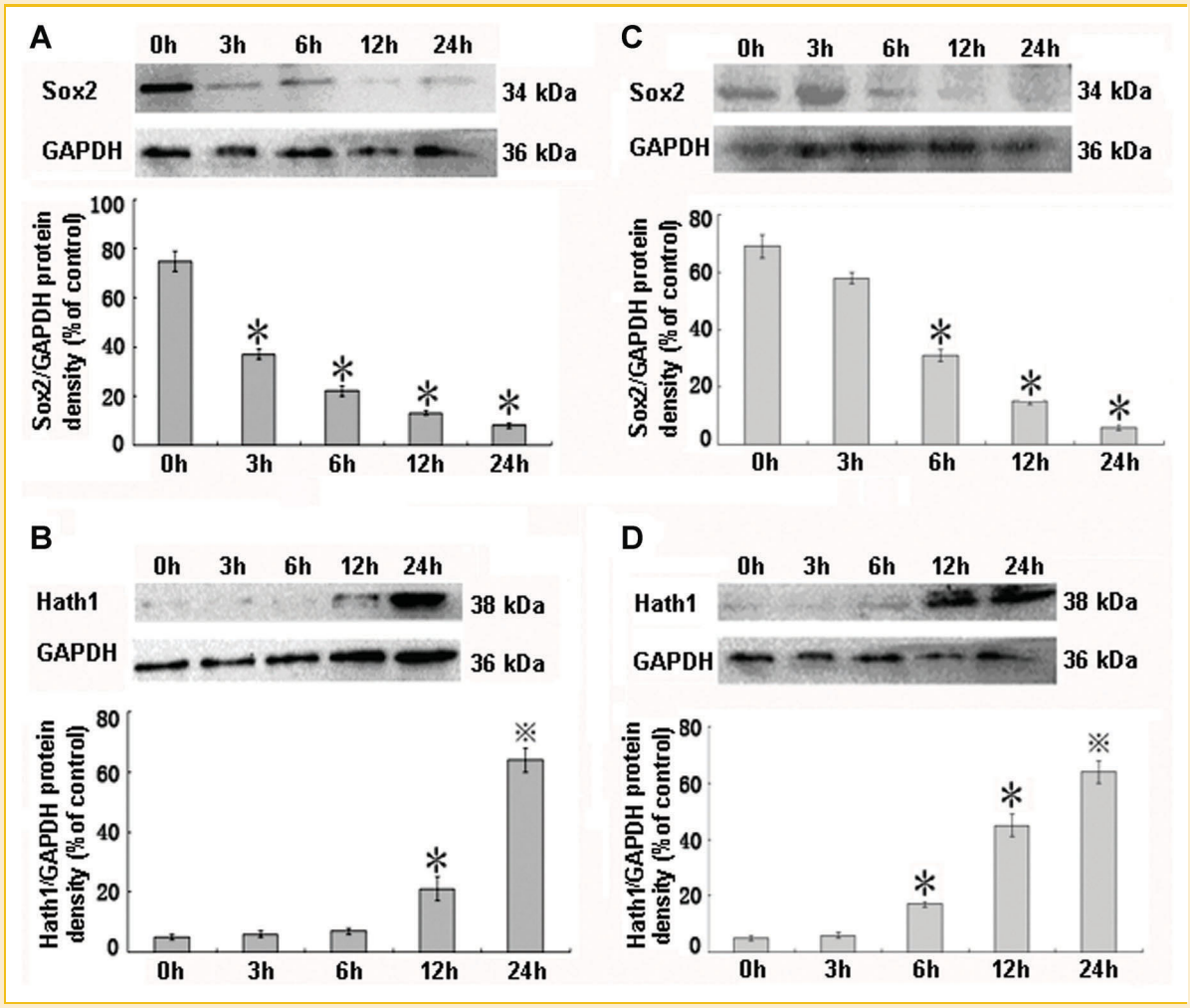

Fig. 2. A viable strain of *H. pylori* (ATCC26695) and a ΔCagA strain (the standard isogenic mutant strain of ATCC43579) down-regulated Sox2 and up-regulated Hath1 expression. After stimulation with a viable strain of *H. pylori* (ATCC26695, A,B) or a viable ΔCagA strain (the standard isogenic mutant strain of ATCC43579, C,D) at various times, MKN45 (A,C) and MKN28 (B,D) whole cell proteins were extracted and analyzed with an immunoblotting method using Sox2 (A,C) and Hath1 (B,D) antibodies, respectively. GAPDH expression was used as an internal loading control. Densitometric analyses for the band densities of Sox2 and Hath1 over GAPDH protein were performed. The graphs represent the means ± SD of three separate experiments. The error bars represent the SD. $^{*}P < 0.05$ relative to the control cells. ※$P < 0.01$ relative to the control cells.

the cells were infected with viable CagA− *H. pylori*. These results indicated that the up-regulation of Hath1 expression by *H. pylori* was independent of the presence of CagA.

HATH1 EXPRESSION STIMULATED WITH *H. pylori*-RELATED CYTOKINES

Because the mechanism of Sox2 down-regulation by *H. pylori* has been proven by the Imatani group [Asonuma et al., 2009], our study focused on the regulation of Hath1 expression in *H. pylori*-infected gastric epithelial cells. The chronic inflammation of the gastric mucosa due to *H. pylori* was related to the host immune response, which resulted in the production of proinflammatory cytokines, such as IL-1β, IL-4, IL-6, IL-8, IFN-γ, and TNF-α. To determine whether Hath1 expression would be altered by IL-1β, IL-6, and IL-8 that were produced by the *H. pylori* infection, MKN28 cells were stimulated with an array of IL-1β, IL-6, and IL-8 concentrations at different time points. No changes in Hath1 expression were observed with the use of IL-1β and IL-6 at different concentrations (Fig. 3A,C) and different incubation times (Fig. 3B,D). However, IL-8 enhancement of Hath1 expression in MKN28 cells was dose- (Fig. 3E) and time-dependent (Fig. 3F). Notably, *H. pylori* with or without the CagA gene stimulated Hath1 expression more significantly than IL-8 in the MKN28 cells (Figs. 2B,D and 3E,F). To further confirm the role of cytokines in *H. pylori*-induced Hath1 expression, MKN28 cells were pre-treated with IL-6 and IL-8 blocking antibodies (R&D Systems), followed by ΔCagA *H. pylori* stimulation for 24 h. IL-8 antibodies significantly inhibited ΔCagA *H. pylori*-induced Hath1 expression, whereas IL-6 had no apparent effect on Hath1 expression (Fig. 3G). Consistent with the observation that IL-8 plays a role in *H. pylori*-induced Hath1 expression, we also detected significant increases in IL-8 levels in the culture supernatants of the MKN28 cells after stimulation with the ΔCagA strain in a time-

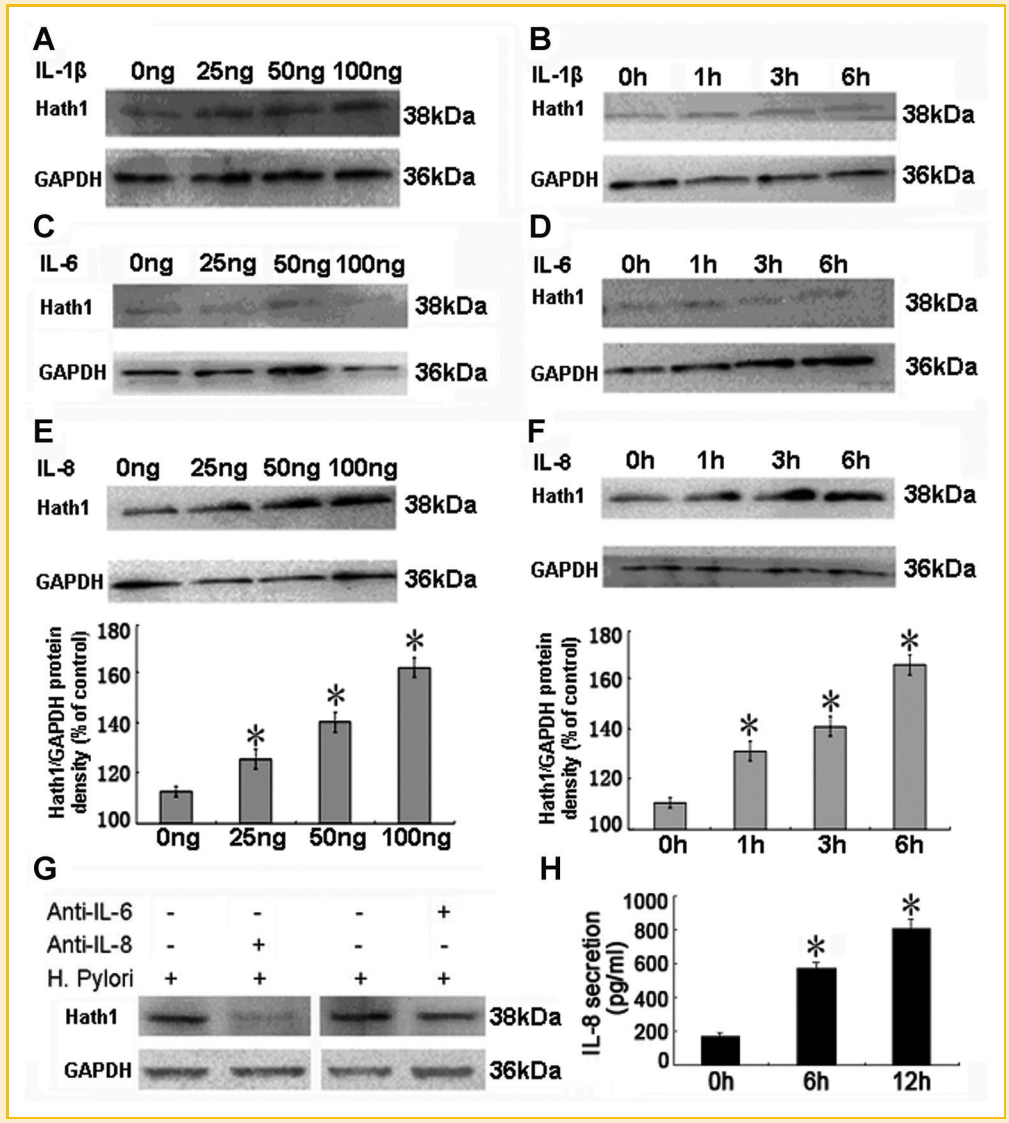

Fig. 3. The effect of *H. pylori*-related cytokine stimulation on Hath1 expression in MKN28 cells. MKN28 cells were stimulated with *H. pylori*-related cytokines IL-1β (A,B), IL-6 (C,D), and IL-8 (E,F) at different concentrations and time points. After stimulation, MKN28 whole cell proteins were extracted and analyzed with an immunoblotting method using an anti-Hath1 antibody. The MKN28 cells were pre-treated with IL-6 and IL-8 blocking antibodies, followed by *H. pylori* stimulation for 24 h. The IL-8 antibodies significantly inhibited ΔCagA *H. pylori*-induced Hath1 expression, whereas IL-6 had no apparent effect on Hath1 expression (G). ΔCagA *H. pylori* greatly induced IL-8 secretion in MKN28 cells at different time points (H). Densitometric analysis for the band density of Hath1 over GAPDH protein was performed. The graphs represent the means ± SD of three separate experiments. The error bars represent the SD. $^{*}P < 0.05$ relative to the control cells.

dependent manner (Fig. 3H). These findings suggest that *H. pylori*-induced IL-8 was involved in Hath1 expression.

H. pylori-INDUCED HATH1 EXPRESSION WAS RELATED TO HES1 INHIBITION

Hes1 is a repressor of Hath1 expression [Zheng et al., 2011]. To explore whether elevated Hath1 expression induced by the *H. pylori* infection was related to Hes1 suppression, Hes1 expression levels in MKN28 cells incubated with viable CagA+ *H. pylori* and ΔCagA *H. pylori* were analyzed. In a time-dependent manner, Hes1 expression levels were significantly decreased after MKN28 cells were incubated with viable CagA+ *H. pylori* (Fig. 4A) and ΔCagA *H. pylori* (Fig. 4B). We considered whether *H. pylori*-induced Hes1 inhibition be mediated by proinflammatory cytokines, such as IL-1β, IL-6, and IL-8. However, no changes were found in Hes1 expression in MKN28 cells incubated with IL-1β (Fig. 4C), IL-6 (Fig. 4D), and IL-8 (Fig. 4E) at various concentrations. Hes1 is a direct downstream target gene of the Notch signaling pathway [Helms et al., 2000]; thus, we evaluated the influence of *H. pylori* and *H. pylori*-related cytokines on Notch signaling activation. The plasmid pGa981-6, which contains six copies of the RBP-J binding motif within the luciferase promoter, was used to detect Notch signaling activity [Qin et al., 2004]. Consistently, *H. pylori* strains, with or without the CagA gene, significantly suppressed Notch activation, whereas IL-6 and IL-8 had no apparent influence on Notch activity at a concentration of 50 ng/ml (Fig. 4F). These results

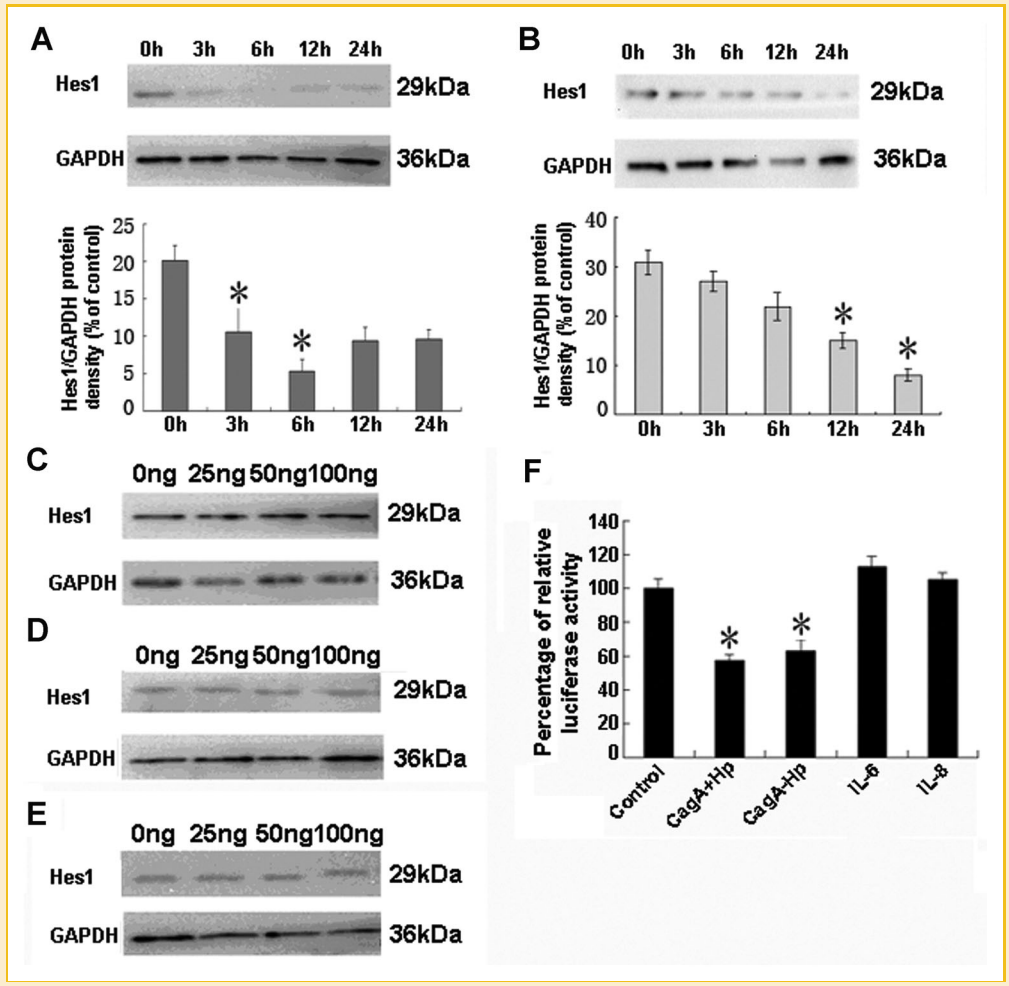

Fig. 4. *H. pylori*, but not *H. pylori*-related cytokines, suppressed Hes1 expression. Hes1 protein was analyzed by immunoblotting the extracted MKN28 whole cell proteins after infection with viable CagA+ *H. pylori* (A) and ΔCagA *H. pylori* (B) at various time points and after stimulation with various concentrations of IL-1β (C), IL-6 (D), and IL-8 (E). Densitometric analysis for the band density of Hes1 over GAPDH protein was performed. Notch signaling activity was detected with the RBP-J-responsive luciferase reporter plasmid in MKN28 cells that were inhibited after infection with viable CagA+ *H. pylori* and ΔCagA *H. pylori*. However, IL-6 and IL-8 at concentrations of 50 ng/ml had no apparent effect on the Notch signaling activity in MKN28 cells (F). For all of the above results, the graphs represent the means ± SD of three separate experiments. The error bars represent the SD. $^{*}P < 0.05$ relative to the control cells.

indicated that *H. pylori*-induced Hes1 inhibition was not mediated by the proinflammatory cytokines IL-6 and IL-8.

IL-8 LED TO STAT3 PHOSPHORYLATION IN MKN28 CELLS

As shown in Figure 5, the increased expression of phosphorylated STAT3 was dose-dependently observed within 6 h of IL-6 stimulation, but the levels of non-phosphorylated STAT3 were unchanged although under IL-6 stimulation (A1 and A2). The increased expression levels of both phosphorylated and non-phosphorylated STAT3 were dose-dependently observed within 6 h of IL-8 stimulation (B1 and B2).

To verify whether an *H. pylori* infection induced STAT3 phosphorylation in the human gastric mucosa, immunohistochemistry and Western blotting methods were used to measure STAT3 activity in 35 gastric mucosal specimens that were obtained from gastritis patients who underwent endoscopy before and 2 months after *H. pylori* eradication. The mucosal IL-8 and IL-6 levels were also detected through ELISA. Phospho-STAT3 staining was more strongly expressed in the gastric epithelial cell nuclei in the *H. pylori*-infected gastric mucosa compared with specimens after *H. pylori* eradication (Fig. 6A), which suggests that STAT3 phosphorylation was partially abrogated by the removal of the *H. pylori* infection. Mucosal IL-8 and IL-6 levels of were significantly lower after *H. pylori* eradication (Fig. 6B). We then analyzed the relationship between the levels of these two cytokines and the level of phosphorylated STAT3; the mucosal levels of IL-8 and IL-6 were closely correlated with levels of phospho-STAT3 before and after *H. pylori* eradication (Fig. 6C).

HATH1 EXPRESSION WAS INHIBITED BY THE INHIBITION OF STAT3 PHOSPHORYLATION

To further confirm whether IL-8-mediated STAT3 signaling can regulate Hath1 expression, tyrphostin AG490, a JAK specific inhibitor that significantly inhibits the expression of phospho-

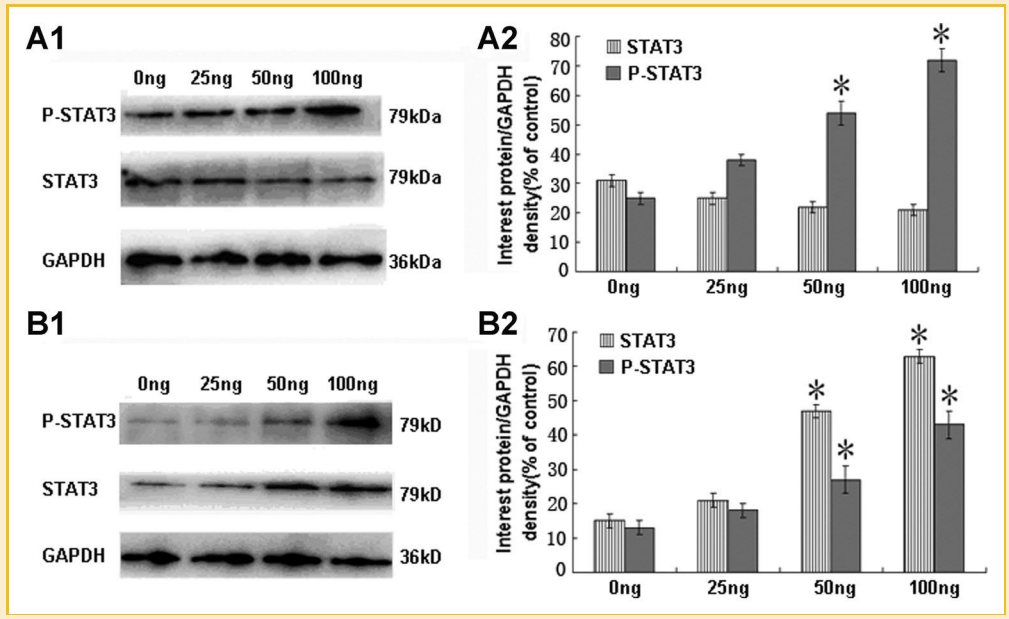

Fig. 5. Both IL-6 and IL-8 induced STAT3 phosphorylation in MKN28 cells. MKN28 cells were stimulated with various concentrations of IL-6 (A1,A2) and IL-8 (B1,B2). Whole cell proteins were then extracted and analyzed with an immunoblotting method using anti-phospho-STAT3 (Tyr705) and anti-STAT3 antibodies. GAPDH expression was used as the control. Densitometric analyses for the band densities of p-STAT3 and STAT3 over GAPDH protein were performed. The graphs represent the means ± SD of three separate experiments. The error bars represent the SD. $^{*}P < 0.05$ relative to the control cells.

STAT3, was added to MKN28 cells at a concentration of 50 μmol/L prior to incubation with different IL-8 concentrations. The expressions of Hes1, Hath1, STAT3, and phospho-STAT3 were detected in the MKN28 cells. As shown in Figure 7A, the STAT3 levels in cells treated with IL-8 (50 or 100 ng/ml) were significantly higher than in cells treated with IL-8 (0 or 25 ng/ml; $P < 0.05$). The phospho-STAT3 levels were almost undetectable in all groups, and Hath1 expression was not increased under the stimulation of IL-8 in the presence of AG490 compared with untreated cells. There was no significant difference in the level of Hes1 in any group.

To specifically suppress STAT3 activity, an RNA interference experiment was performed with a shRNA-STAT3 plasmid. STAT3 protein expression was greatly reduced (Fig. 7B). There was almost no detectable phospho-STAT3, even with stimulation of IL-8. Similarly, in addition to the inhibition of STAT3 by RNA interference, Hath1 expression was also undetectable even with the stimulation of different concentrations of IL-8.

DISCUSSION

IM is characterized by a normal gastric mucosa that is replaced by a type of mucosa that resembles the human intestine [Busuttil and Boussioutas, 2009]. The de novo expression of intestinal MUC2 is common to all types of IM and is accompanied by the gradual loss of gastric mucin markers [Tatematsu et al., 2003; Flejou, 2005]. Although the corresponding regulators of aberrant gastric epithelial cell differentiation are not well known, evidence suggests that several transcription factors, such as CDX2, Sox2, PDX1, OCT-1, RUNX3, and sonic hedgehog, are involved in this process [Fukamachi et al., 2004; Almeida et al., 2005; Tsukamoto et al., 2005; Leys et al., 2006; Park do et al., 2010]. Intestinal goblet cells are developed in the gastric fundus of CDX2-transgenic mice [Silberg et al., 2002]. These studies suggest that a pathologically common molecular feature of IM is the up-regulation of intestinal epithelium terminal differentiation factors concomitant with the down-regulation of gastric epithelium terminal differentiation factors. Our previous research has consistently shown that Hath1, a master regulator of secretory cell (i.e., goblet, enteroendocrine, and Paneth cells) fate commitment in the intestinal epithelium, was up-regulated in well-differentiated gastric cancer tissue [Zhang et al., 2010].

Many previous researchers have indicated that *H. pylori* virulence factors can induce aberrant differentiation of gastric epithelial cells. One recent example is that of the *H. pylori* CagA region that triggers gastric epithelial cells to express bactericidal lectin REG3γ that is normally expressed by Paneth cells at the distal small intestine via IL-11/gp130/STAT3 activation [Lee et al., 2012]. This example suggests that *H. pylori* is associated with the phenotypic transformation of gastric epithelium cells into Paneth cells. In the present study, we found that co-cultured *H. pylori* with gastric epithelial MKN28 and MKN45 cells led to up-regulated Hath1 expression and down-regulated Sox2 expression, respectively, which elucidated a new molecule mechanism connecting *H. pylori* infection and IM development. Previous researchers have found that *H. pylori* virulence factors, especially CagA protein, lead to oxidative stress in the stomach, which causes elevated reactive oxygen species (ROS) production or a decline in antioxidant defenses of host cells. Moreover, CagA-induced pathological signal transduction plays an important role in *H. pylori*-induced gastric

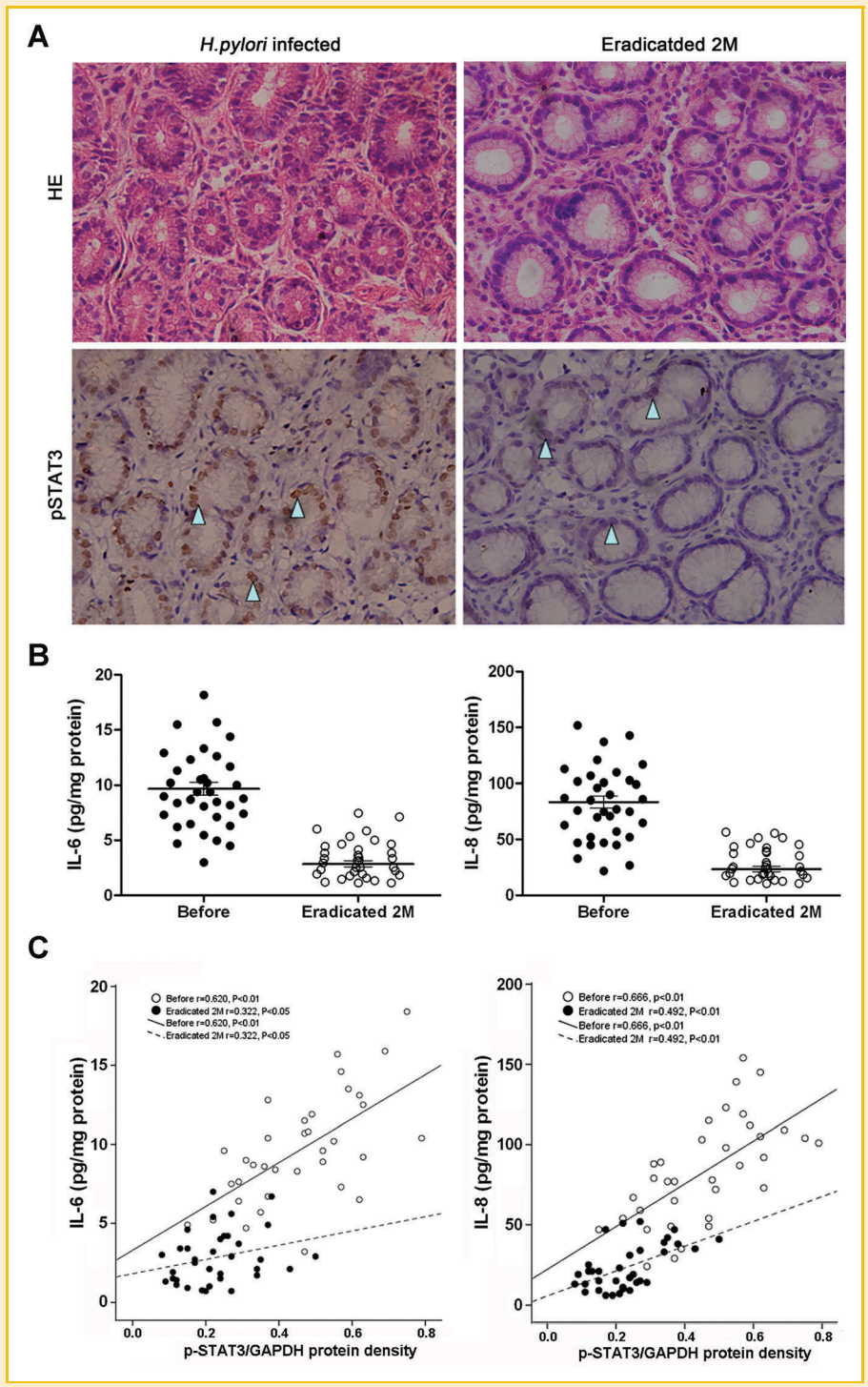

Fig. 6. IL-6 and IL-8 levels correlated with the phosphorylation of STAT3 in the *H. pylori*-infected human gastric mucosa. Representative images of gastric mucosal tissues that underwent HE staining and immunohistochemical staining of phospho-STAT3 (Tyr-705) before and 2 months after *H. pylori* eradication (A) in which phospho-STAT3 (Tyr-705) was observed in the nuclei of gastric mucosal epithelial cells (marked as arrowhead). IL-6 and IL-8 levels in the *H. pylori*-infected human gastric mucosa were significantly reduced 2 months after *H. pylori* eradication as detected by the ELISA method ($P < 0.05$, B). Both IL-6 and IL-8 levels correlated with phospho-STAT3 levels in the *H. pylori*-infected human gastric mucosa before and 2 months after *H. pylori* eradication (C). Phospho-STAT3 was calculated with densitometric analysis for the band density of phospho-STAT3 over GAPDH protein. Correlation coefficients were calculated with the Spearman's rank correlation test.

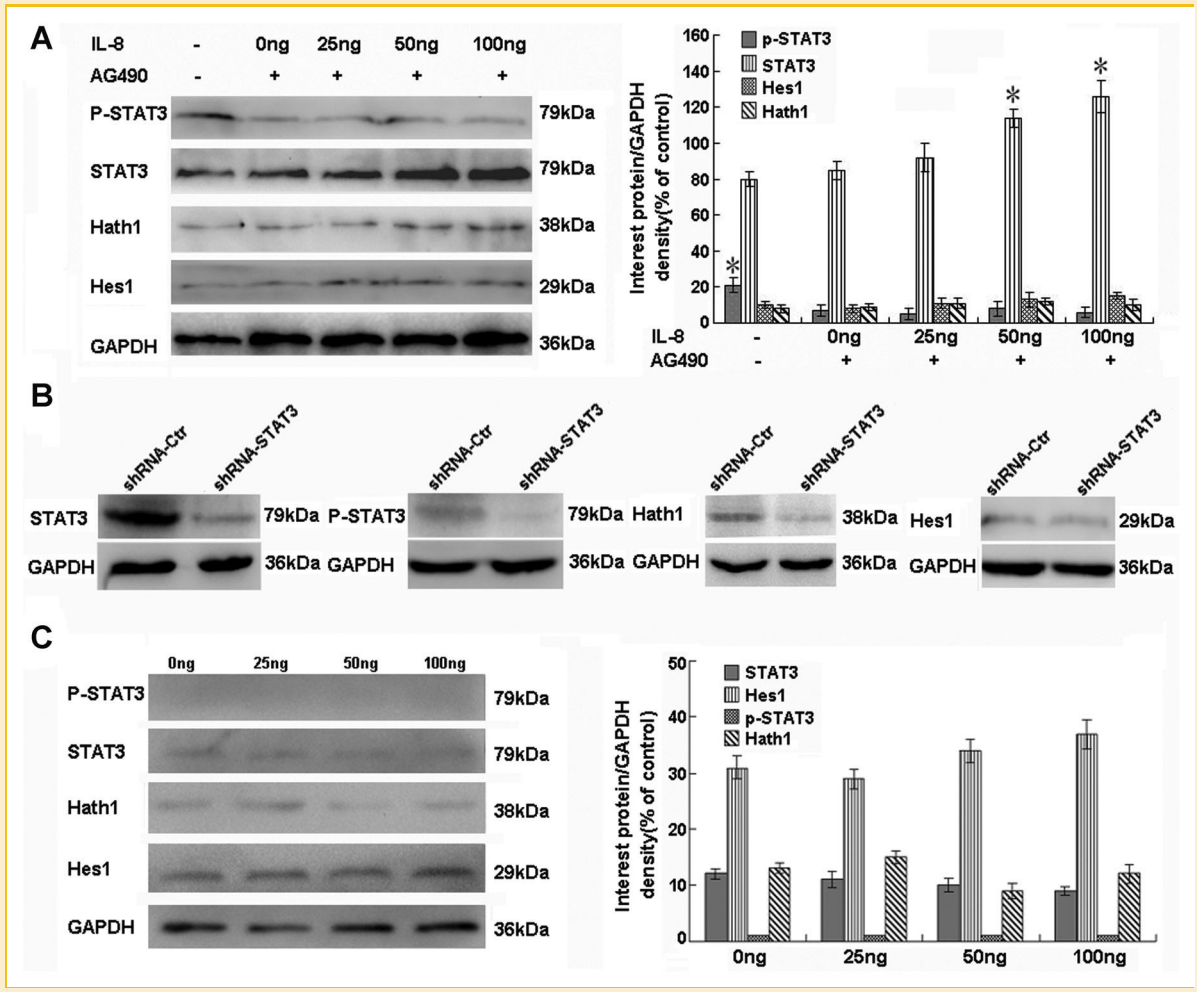

Fig. 7. The suppression of STAT3 activation abolished IL-8-induced Hath1 protein expression. The STAT3 phosphorylation inhibitor, tyrphostin AG490, was added 30 min prior to the addition of IL-8 to the cultured MKN28 cells. Whole cell proteins were then extracted and analyzed with an immunoblotting method using the respective antibodies (A). Comparative expression of STAT3, p-STAT3, Hath1, and Hes1 between pRNAT-U6.1-shRNA-STAT3/MKN28 cells and pRNAT-U6.1-shRNA-Ctr/MKN28 cells were detected by Western blot (B). The selected pRNAT-U6.1-shRNA-STAT3/MKN28 cells, in which STAT3 expression had been suppressed, were subjected to stimulation with various concentrations of IL-8. Whole cell proteins were then extracted and analyzed with an immunoblotting method using the respective antibodies (C). Densitometric analyses for the band densities of p-STAT3, STAT3, Hes1, and Hath1 over GAPDH protein were performed. The graphs represent the means ± SD of three separate experiments. The error bars represent the SD. $^{*}P < 0.05$ relative to the control cells.

carcinogenesis [Handa et al., 2007; Suzuki et al., 2012]. Interestingly, in the present study, we found that *H. pylori* induced up-regulation of Hath1 expression in MKN28 cells and down-regulation of Sox2 expression in MKN45 cells was CagA protein independent, which implies that other virulence factors associated with *H. pylori* also play an essential role in *H. pylori*-induced aberrant differentiation of the gastric epithelium.

To characterize the role that *H. pylori*-related cytokines played in *H. pylori*-induced Hath1 expression, we found that the proinflammatory cytokine IL-8, but not IL-1β or IL-6, was partially responsible for elevated Hath1 expression in MKN28 cells. Many components of *H. pylori* employ varieties of mechanisms to induce IL-8 production. For example, OipA, a virulence factor outside of Cag PAI, induces IL-8 release through PI3K/Akt signaling and depends on the signaling downstream transcription factors FoxO1/3a, whereas PAI-mediated IL-8 production employs FoxO1/3-independent signaling [Tabassam et al., 2012]. Although *H. pylori* with a CagA region, especially tyrosine within the C-terminal phosphorylated CagA domain, was more potent in inducing IL-8 production [Lai et al., 2011], which was consistent with previous reports [Takagi et al., 1997], we found that the ΔCagA strain also greatly induced IL-8 production in gastric epithelial cells. This finding is concordant with the conclusion that *H. pylori* up-regulates Hath1 expression through inducing the release of IL-8.

Our research provided further evidence that the mucosal levels of *H. pylori*-related cytokines IL-6 and IL-8 were significantly higher in the *H. pylori*-infected specimens. After the eradication of *H. pylori*, the gastric mucosal levels of IL-8 and IL-6 were significantly decreased; both IL-8 and IL-6 expression levels correlated positively with phosphorylated STAT-3 before and after *H. pylori* eradication. IL-6 and IL-8 could induce STAT3 phosphorylation in a concentration-dependent manner in MKN28

cells. Interestingly, only IL-8 induced Hath1 expression. The results from AG490 inhibition and shRNA-mediated silencing of STAT3 suggested that STAT3 activation regulates Hath1 expression. Knowing that the IL-8-neutralizing antibody, but not the IL-6-neutralizing antibody, blocked *H. pylori*-induced Hath1 expression, we concluded that *H. pylori*-induced Hath1 expression was IL-8/STAT3 pathway dependent. This conclusion is consistent with previous reports [Ando et al., 1998] that IL-6 signals via gp130 and thus, activates a variety of signaling molecules, such as STAT1 and STAT3, tyrosine phosphatase SHP-2, and mitogen-activated protein kinases (MAPK) ERK1 and ERK2 [Neurath and Finotto, 2011], whereas IL-8 signaling is mediated through the binding of IL-8 to two cell-surface G protein-coupled receptors, CXCR1, and CXCR2, which subsequently activates phosphatidyl-inositol-3-kinase, PKC, and Rho GTPase family [Waugh and Wilson, 2008]. There was a discrepancy between our result that the ΔCagA strain could induce STAT3 activation with previous reports that *H. pylori*-induced STAT3 phosphorylation was strictly dependent on the presence of CagA gene [Bronte-Tinkew et al., 2009; Lee et al., 2010]. We interpret the different genetic backgrounds of the different stains that were used to be the source of the difference in results. Performing a comparison between these two strains may facilitate our understanding the difference.

Previous studies have shown that the initiation of Math1 (a homolog of Hath1) expression appears to be dependent on bone morphogenetic protein (BMP) signaling [Lee et al., 2000]. Helms et al. [2000] identified a 1.7 kb enhancer located 3′ of the Math1-coding region that could sufficiently drive the specific expression of lacZ in several Math1 expression domains. Later, Ebert et al. [2003] identified a novel-binding site for Zic1 in the Math1 enhancer that was located 30 bp from the auto-regulatory E-box site and found that Zic1 repressed the auto-activation of Math1 expression. Recent research determined that β-catenin up-regulates Atoh1 (a homolog of Hath1) expression in neural progenitor cells by interacting with an Atoh1 3′ enhancer [Shi et al., 2010]. Additionally, Bollrath et al. have suggested that STAT3 activation may enhance nuclear localization of β-catenin [Grivennikov et al., 2009]. This mechanism partially explained why STAT3 activation led to up-regulation of Hath1 expression. Whether STAT3-regulated Hath1 expression occurs through other mechanisms requires further exploration.

We found that *H. pylori*-mediated up-regulation of Hath1 expression accompanied Hes1 suppression in MKN28 cells. Hes1 could suppress Hath1 expression by binding to the 5′ promoter region of Hath1 [Zheng et al., 2011]. Previous research demonstrated that Hes1 facilitated STAT3 phosphorylation through a complex formation with STAT3 [Kamakura et al., 2004]. However, we did not observe increased Hes1 levels during STAT3 phosphorylation with IL-6- and IL-8-stimulated MKN28 cells.

The polymorphism of the IL-8 gene at position −251 (IL-8 −251A/T) resulted in increased IL-8 expression and was associated with the progression of gastric atrophy and metaplasia in *H. pylori*-infected patients [Szoke et al., 2008]. In this study, we identified an abnormal differentiation process that was mediated by IL-8 through the up-regulation of Hath1 expression. Additionally, our study provided further evidence that IL-8-induced STAT3 phosphorylation is at least partially responsible for such a process. Studies have demonstrated that STAT3 activation that results from IL-8/CXCR2 plays a critical role in inducing cell transformation, angiogenesis, and proliferation [Yang et al., 2010]. Improvement of IM by *H. pylori* eradiation was confirmed in IM patients. IM is widely accepted as a pre-cancerous lesion of gastric carcinoma, which suggests that *H. pylori* eradication is a valuable treatment in IM patients. However, the exact molecular mechanisms behind the event are still unclear. This research provides important information on how such signaling pathways regulate the abnormal differentiation of gastric epithelium. It may be feasible to inhibit this pathway, which may lead to new treatments for *H. pylori*-induced gastric metaplasia.

ACKNOWLEDGMENTS

We are grateful to Professor Han Hue (Department of Genetic and Developmental Biology, Fourth Military Medical University, Xi'an, China) for kindly providing us with the pGa981-6 plasmid.

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